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13. ABSTRACT *(Maximum 200 words)* 1,1,1-Trichlorethane (1,1,1-TCA), a widely used industrial solvents, is one of the most common subsurface contaminants. Transformation processes in the subsurface can result in the production of 1,1-dichloroethane (1,1-DCA) and 1,1-dichloroethene (1,1-DCE) from 1,1,1-TCA contamination, resulting in plumes of mixed chlorinated aliphatic hydrocarbons (CAHs). A butane-utilizing microorganism, strain 183BP, with the ability to cometabolically transform 1,1,1-TCA, 1,1-DCA, and 1,1-DCE was isolated from environmental samples taken from a CAH contaminated site. Two bioaugmentation treatment tests with strain 183BP as inoculum were conducted at the Moffett Federal Airfield In-Situ Bioremediation Test Site (Moffett Field), Mountain View, California. Also, a soil column packed with aquifer solids and groundwater obtained from Moffett Field was inoculated with strain 183BP and operated under conditions similar to those used in the field tests.

Field groundwater samples and soil column effluent samples were analyzed using techniques based on 16S rRNA gene analysis. 183BP-specific primers were designed and used in real-time SYBR Green I PCR analyses to detect and quantify the inoculated microorganisms in the subsurface. Dynamics of the bacterial community composition were investigated using terminal restriction fragment length polymorphism (T-RFLP) methods and statistical analysis.

During the first bioaugmentation test in the absence of 1,1-DCE, maximum treatment efficiencies for TCA and DCA were approximately 80% and 96%, respectively in the bioaugmented well leg, while essentially no transformation occurred in the non-bioaugmented control leg. During the effective treatment period, the 183BP cell concentration was above 900 cells/ml in groundwater obtained 0.5 m from the injection well. In the second bioaugmentation test, 1,1-DCE was added to the influent CAH mixture and was effectively transformed in the bioaugmented well leg. Although 93% of the influent 1,1-DCE was transformed, 1,1-DCA and 1,1,1-TCA removal efficiencies were significantly reduced compared to the test in the absence of 1,1-DCE.

In the soil column, maximum treatment efficiencies for TCA and DCE were approximately 96% and 77%, respectively. Microbial results indicated that the decline in TCA concentrations was concomitant to an increase in the concentration of strain 183BP cells. The bacterial community had greater species diversity than field samples and did not follow the same succession trend as the field samples. However, addition of 1,1-DCE in the feed to the column resulted in a similar reduction of 1,1-DCA and 1,1,1-TCA transformation efficiencies as that observed in the field studies.

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Molecular Analysis of Bacterial Community Dynamics During Bioaugmentation Studies in a Soil Column and at a Field Test Site

Final Report

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PREFACE

This report was submitted as a thesis to the Oregon State University as part of Cooperative Research and Development Agreement (CRADA) with the Air Force Materiel Command. Funding was provided by the Strategic Environmental Research and Development Program (SERDP) and the CRADA was administered by the Air Force Research Laboratory, Tyndall AFB, Florida, 32403 under contract number F33615-99-2-5800.

This thesis is being published in its original format by this laboratory because of its interest to the worldwide scientific and engineering community. This thesis covers work performed between October 2003 and June 2004. AFRL/MLQ project was Lt Kolin Newsome.

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Molecular Analysis of Bacterial Community Dynamics During Bioaugmentation Studies in a Soil Column and at a Field Test Site

by

Jun Li

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Presented June 3, 2004

Commencement June 2004

Master of Science thesis of Jun Li presented on June 3, 2004

APPROVED:

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AN ABSTRACT OF THE THESIS OF

Jun Li for the degree of Master of Science in Civil Engineering presented on June 3, 2004.
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Abstract approved:

Mark E. Dolan

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183BP was found to be identical to that of *Rhodococcus sp*. USAN-12 (Genbank accession number AF420413).

Two bioaugmentation treatment tests with strain 183BP as inoculum were conducted at the Moffett Federal Airfield In-Situ Bioremediation Test Site (Moffett Field), Mountain View, CA. Also, a soil column packed with aquifer solids and groundwater obtained from Moffett Field was inoculated with strain 183BP and operated under conditions similar to those used in the field tests.

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reduced compared to the test in the absence of 1,1-DCE. The 183BP cell concentration was almost 1-log-order higher than that of the first test and clear spatial distribution of the cells among the monitoring wells was observed. The bioaugmented strain 183BP was not observed in T-RFLP analyses conducted on groundwater samples during either bioaugmentation test. The groundwater bacterial community profiles were alternately dominated by two peaks, 277-bp during the early stages of amendments and 126-bp during the later stages of both tests.

In the soil column, maximum treatment efficiencies for TCA and DCE were approximately 96% and 77%, respectively. Microbial results indicated that the decline in TCA concentrations was concomitant to an increase in the concentration of strain 183BP cells. The bacterial community had greater species diversity than field samples and did not follow the same succession trend as the field samples. However, addition of 1,1-DCE in the feed to the column resulted in a similar reduction of 1,1-DCA and 1,1,1-TCA transformation efficiencies as that observed in the field studies.

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CHAPTER 1
INTRODUCTION

Groundwater contamination is of significant health concern since it is used as a drinking water supply by many people. 1,1,1-Trichloroethane (1,1,1-TCA) is a man-made halogenated solvent and primarily used as industrial cleaning and degreasing agent. It is one of the common CAH contaminants found in groundwater and soil environments, where it can generate extensive plumes. The maximum contaminant level (MCL) and maximum contaminant level goal (MCLG) of 1,1,1-TCA in drinking water are 0.2 mg/L (EPA, 2002). It is believed that 1,1,1-TCA has the potential to cause liver, nervous system, and circulatory system damage from acute and lifetime exposure at levels above the MCL. Bacterial cultures isolated from various environmental media are capable of degrading 1,1,1-TCA either through aerobic cometabolic or anaerobic pathways (Egli et al, 1987; Galli and McCarty, 1989; de Best et al 1999; Yagi et al., 1999; Kim et al, 1997).

The Moffett Federal Airfield In-Situ Bioremediation Test Site (Mountain View, CA) is one of the sites that were contaminated with various chlorinated aliphatic hydrocarbons (CAH), including 1,1,1-TCA. Remediation assessment studies conducted at Moffett field showed that methane-oxidizing bacteria which use methane as primary substrate and cometabolically transform CAHs with a methane monooxygenase enzyme achieved significant transformation of vinyl chloride (VC) and trans-1,2-dichlroethene (t-DCE), but exhibited limited transformation of 1,1,1-TCA, trichloroethylene (TCE) and cis-1,2-dichlroethene (c-DCE) (Semprini et al., 1990; Semprini et al., 1991). In other field tests (Hopkins et al., 1993; Hopkins and McCarty, 1995), equally effective removal of c-DCE and TCE by phenol- and toluene-oxidizing bacteria whose toluene oxygenase enzyme

is responsible for CAHs cometabolism were observed, but t-DCE was least transformed. However, in microcosm studies none of microcosms effectively degraded 1,1,1-TCA when fed phenol, toluene, methane or ammonia (Hopkins et al, 1993; Hopkins and McCarty, 1995). Fries et al (1997) identified 63 strains from 273 phenol- and toluene-degrading isolates that grew in a Moffett Field, CA., aquifer and found that most of them can cometabolize TCE , which indicates that there a significant number of naturally occurring strains may support successful phenol- and toluene-stimulated TCE transformation.

Bioaugmenting microorganisms with known degradative abilities may improve biotransformation of chlorinated aliphatics at Moffett Field. Several bioaugmentation treatment tests conducted at contaminated sites resulted in successful transformation the targeted toxins to harmless compounds (Baud-Grasset et al, 1995; Fantroussi et al, 1999; Major et al, 2002; Salanitor et al, 2000). Kim et al (1997) enriched a mixed community from a CAH-contaminated DOE site in Hanford, WA. The enrichment culture was able to cometabolize 1,1,1-TCA, 1,1-DCE and a number of other CAHs using butane as a primary substrate. A butane-utilizing organism was isolated from the enrichment and was shown to have 1,1,1-TCA, 1,1-DCA, and 1,1-DCE transformation abilities (Mathias, 2001). Characterization of the 16S rRNA gene of the organism was found 100% identical to a known *Rhodococcus sp*. USAN-12 (Genbank accession number AF420413). During the past few years, a number of field bioaugmentation pilot tests with cultures containing strain 183BP have been conducted at the Moffett field. The ability of the inocula to survive, adapt and flourish in a non-native environment and function well in biodegradating the targeted contaminants is an important concern in bioaugmentation. In addition, in the present study a continuous-flow soil column was constructed with groundwater and aquifer solids collected during the installation of wells for the field tests and was operated and sampled on a routine basis as a comparison to field observations.

Microbial analysis may provide a critical key in understanding the contaminant biodegradation process. Traditional approaches like culture plating and the most probable number (MPN) method are often too selective, time-consuming and inaccurate. The development of molecular-based techniques allows detecting and/or quantifying microorganisms in different environments using 16S rDNA/rRNA genes (Hugenholz et al, 1998; Hendrickson et al 2002; Dojka et al, 1998; Fennell et al 2001; Major et al, 2002; Löeffler et al., 2000; DeLong et al., 1999).

To date, bioaugmentation treatment tests are always critically assessed in terms of efficiency and effectiveness. In most cases, the inocula are usually highly active and efficient in removal of the contaminants under laboratory conditions; whereas, it is difficult to predict their performance under natural conditions, and very little is known about actual cell densities and spatial distribution over the duration of the bioaugmentation treatment test. Hence, the objective of this research was to develop a quantitative SYBR Green I real-time PCR assay based upon 16S rRNA genes to provide assessment of the abundance and role of an augmented culture (stain 183BP) in 1,1,1-TCA biodegradation tests conducted at the Moffett field, CA as well as in a soil column, and to characterize the bacterial community structure and possible community shifts corresponding to 1,1,1-TCA biodegradation using terminal restriction fragment length polymorphism (T-RFLP) methods and statistical analyses.

CHAPTER 2
LITERATURE REVIEW

2.1 Molecular biological techniques

In the kingdom of microorganisms, only a remarkably small portion can be cultured using standard culturing techniques (e.g., traditional plate methods). Furthermore, cultured-based methods are time consuming and are often too selective, particularly for fastidious or yet-to-be cultured bacteria, and therefore this approach does not reflect the exact composition of mixed bacterial communities or microbial diversity (Moter et al., 2000).

Over the past two decades, some molecular techniques based upon ribosomal RNA (rRNA) / DNA (rDNA) genes have been developed to study microbial diversity and ecology, including polymerase chain reaction (PCR), Real-Time PCR, terminal restriction fragment length polymorphism (T-RFLP), and fluorescence in situ hybridization (FISH) etc. Without a necessity of culturing microorganisms, these approaches have revolutionarized microbiological research in terms of detection sensitivity and bacteria identification.

2.2 Ribosomal RNA and phylogeny

Woese, an originator of microbial phylogenetic approaches has stated that "*Ribosomal RNA is a magic molecule*" (Woese, 1995). Residing in ribosomes, rRNA plays a key role in protein synthesis in microorganisms. rRNA also serves as a phylogenetic indicator in light of the following reasons enlisted by Olsen et al. (1986):

> "*1. The rRNAs… are functionally and evolutionarily homologous in all organisms.*

2. *The rRNAs are ancient molecules and are extremely conserved in overall structure.*
3. *Nucelotide sequences are also conserved…the highly conserved regions also provided convenient hybridization targets for cloning the rRNA genes and for primer-directed sequencing techniques.*
4. *The rRNAs consitute a significant component of the cellular mass, and they are readily recovered from all types of organisms for accumulation of a database of reference sequences.*
5. *The rRNAs provide sufficient sequence information to permit statistically significant comparisons.*
6. *The rRNA genes seem to lack artifacts of lateral transfer between contemporaneous organisms. Thus, relationships between rRNAs reflect evolutionary relationships of the organisms.* "

There are three rRNAs in bacteria, the 5S (~120 nucleotides), 16S (~1600 nucleotides) and 23S (~3000 nucleotides) subunits. In most eubacteria, rRNA genes follow the order 5'-16S-23S-5S-3' and are cotranscribed (Gray and Schnare, 1996). Due to the appropriate size required for phylogenetic information and availability of current databases for comparative sequence analysis (e.g., Ribosomal Database Project II [RDP II] and National Center for Biotechnology Information [NCBI]), 16S rRNA is the most favorable molecule to use for phylogenetic analysis.

Based upon the evolution history of microorganisms and their genetic characteristics, phylogeny is a classification method that detects the differences in microorganisms (Rittmann and McCarty, 2001). Usually, the phylogenetic relationship among the organisms is represented by a phylogenetic tree, which begins at a "root" and through a series branchings gives rise to a set of contemporary organisms (Olsen, 1988). There are many methods of inferring a phylogenetic tree from sequence data, including cluster analysis, maximum parsimony, distance matrix, etc. (Olsen, 1988; Woese, 1987). A typical phylogenetic tree is shown in Figure 2.1.

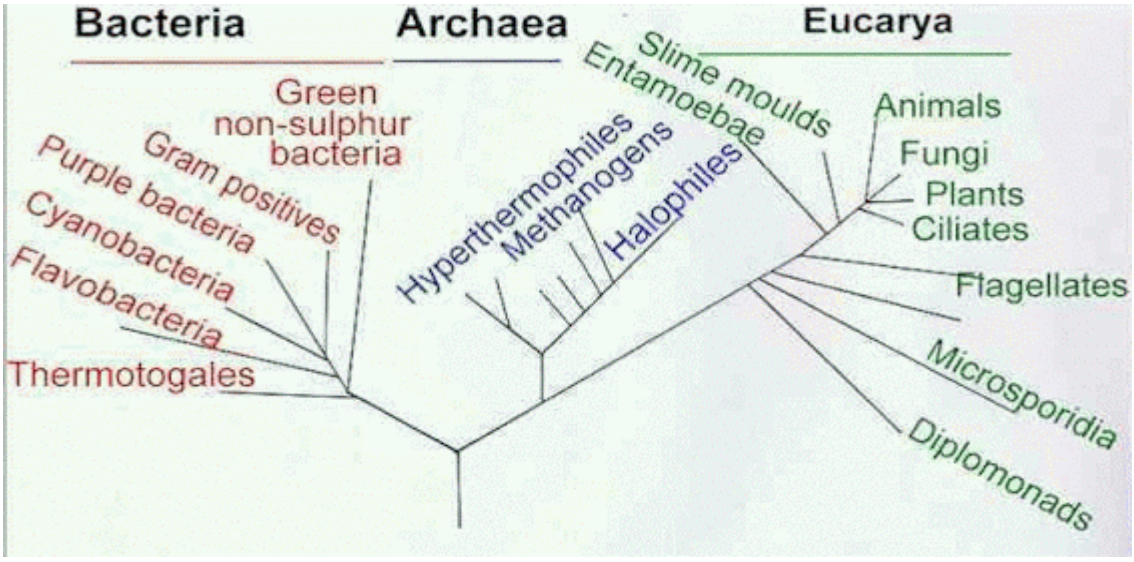


Figure 2.1 Phylogenetic tree of life as determined from comparative rRNA sequencing. Source: Olsen and Woese, 1993.

2.3 Fluorescent in situ hybridization

Fluorescent in situ hybridization (FISH) detects nucleic acid sequences by using a fluorescently labeled probe that hybridizes specifically to its complementary target sequence within an intact cell by using epifluorescence microscopy or confocal laser scanning microscopy (DeLong et al., 1989; Amann et al., 1990; Lipski et al., 2001). Oligonucleotide probes, complementary to target rRNA sequences, commonly contain 15-25 nucleotides. Shorter probes have higher chance to penetrate into cells and bind to their target sequences, but may be of low specificity. Longer probes have greater specificity but can be difficult to transport into the cells. Often, probe sequences can be automatically generated by computer programs, or retrieved directly from oligonucleotide probe databases. However, existing probes should be used cautiously since they were developed from old data sets of rRNA sequences (Amann and Ludwig, 2000; Alm et al., 1996). Generally, there are many thousands of strands of rRNA residing in a single cell, offering

sufficient targets for probes with fluorescent dye to pair up and be visualized by microscopy. The microorganisms targeted by a hybridization probe varies dependent on the region of the molecule chosen as the hybridization target: subspecies- or strain- specific probes match the most variable sequence regions, while more general probes match the more conserved regions (Stahl and Amann, 1991). Commonly universal oligonucleotide probes often are used to quantify total microbial population represented in environmental samples and can also be applied to normalize the results acquired with probes targeting more specific phylogenetic groups of microorganisms (Zheng et al., 1996; Alfreider et al., 1996; Zarda et al., 1997; Christensen et al., 1999; Cottrell et al., 2000; Daims et al., 2001). Oda et al. (2000) designed probes for *Rhodopseudomonas palustris* spp. to examine influence of growth rate and starvation on FISH performance. Yang and Zeyer (2003) developed oligonucleotide probes to detect *Dehalococcoides ethenogenes* present in environmental samples where complete dehalogenation from tetrachloroethene to ethene was found.

There are two basic staining methods for probes. One can either directly label the probe using a fluorescent dye molecule at either the 5' end or 3' end of an oligonucleotide or internally, or attach a reporter group to the probe and detect this probe with a labeled binding protein (Stahl and Amann, 1991). The most important fluorescent compounds used to label oligonucleotides are fluorescein and various fluorescein analogs. Fluorescein is a multi-ring aromatic compound that is strongly fluorescent. Bouvier and Giorgio (2002) quantatively reviewed published reports on FISH using the universal bacterial EUB338 probe and found that probes labeled with the dye Cy-3 generated the highest estimated mean percentage of cells detected.

A common FISH procedure includes four steps: (i) cell fixation; (ii) hybridization; (iii) washing off unbound and non-specifically bound probes; and (iv) visualization (Amann et al., 1990; Stahl and Amann, 1991; Alfreider et al.,

1996; Ouverney and Fuhrman, 1997; Zarda et al., 1997; Davenport et al., 2000; Cottrell and Kirchman, 2000; Crocetti et al., 2000; Daims et al., 2001; Morris, 2002). Cell fixation is generally conducted using formadehyde or paraformadehyde (PFA) solution. Fixation interrupts cellular activities, protects rRNA from degradation, and permeates the cell membrane to allow the probe to penetrate into the cell so that hybridization can occur. However, this treatment has not always been effective with gram positive bacteria due presumably their dense outer cell structure. Additional pretreatment with enzymes and/or chemicals may be necessary to increase permeability of gram-positive bacteria. A 1-min PFA (3% final concentration) fixation (De Los Reyes et al., 1997), 50% ethanol fixation (De Los Reyes et al., 1997; Hugenholtz et al., 2001), enzyme treatment including mutanolysin and lysozyme treatment (Rodrigues, 2003), and lipase and proteinase K treatment (Carr, 2004), mild acid hydrolysis (Macnaughton et al., 1994; Davenpot et al., 2000; Carr, 2004), and a combination of acid hydrolysis and enzyme treatment (Carr, 2004) have achieved variable successes in permeating different microorganisms. In this study, strain 183BP was believed to be a *Rhodococcus sp*. from 16S rRNA gene analysis. Rhodococcus bacteria belong to the mycolic acid-containing actinomycetes (the mycolata). However, representatives of all genera of the mycolic-acid-containing gram-positive bacteria were not tested in all of these studies, and no single permeabilization method was reported which successfully permeabilized all strains of all the genera investigated in these reports.

After the cells are fixed, they are transferred to glass slides and hybridized with probes in a preheated hybridization buffer. Optimal hybridization temperature is determined experimentally and is critical to ensure target specificity, maximum probe signal, and to minimize non-specific hybridizations. In some cases, formamide is used to lower the melting temperature by weakening the hydrogen bonds between DNA strands (Stahl and Amann, 1991; Zarda et al., 1997; Sekiguchi et al., 1999; Crocetti et al., 2000). Often the proportion of target cell to total microbial populations is described, if so, total cell count is also performed using the nucleic acid stain 4',6'-diamidino-2-phenylindole (DAPI) (Alfreider et al., 1996; Davenport et al., 2000; Lipski et al., 2001; Richardson et al., 2002). Since DAPI targets DNA in the cell nucleus and emits fluorescence in a wavelength different from that of the fluorescent dye of rRNA probe, FISH-labeled cells can be distinguished from the DAPI-stained cells by changing fluorescent filters.

After hybridization, the slides are rinsed with washing buffer to remove unbound probes. Post-hybridization stringency can also be adjusted by varying the washing temperature and salt concentration in the washing buffer. Finally, slides are air dried in the dark and mounted. Usually, the mounting media are anti-photofading agents (e.g., citifluor).

Then the slides are visualized by equipment which can detect fluorescence-stained cells. Flow cytometry can be used to count cell number by optical methods. In flow cytometry, cell suspensions in a continuous fine stream pass through laser beam and scatter some of the laser light and emit fluorescence which can be captured and recorded by specific detectors (Lipski et al., 2001). The fluorescence can also be microscopically imaged and stored using a digital camera. Epifluorescence microscopy and confocal laser scanning microscopy (CLSM) are excellent tools for this purpose. Epifluorescence microscopes have very bright light sources. "Epi" fluorescence refers to the design of the microscope. Instead of the condenser focusing the exciting light onto the specimen and the objective collecting the emitted light, there is only one lens for both tasks, the dichromtic beam splitter.

Generally, in conventional light microscopy, all light passing through a specimen is imaged directly and simultaneously

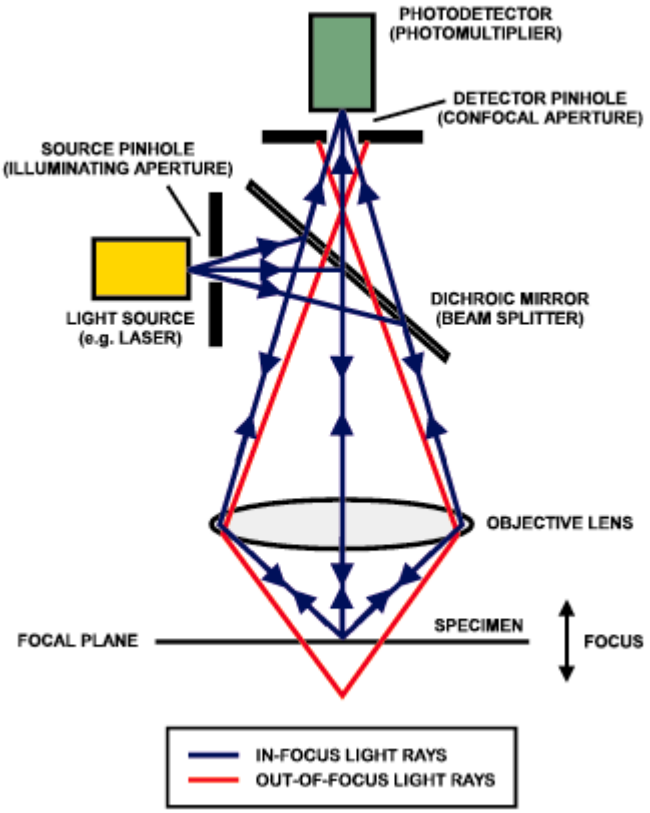


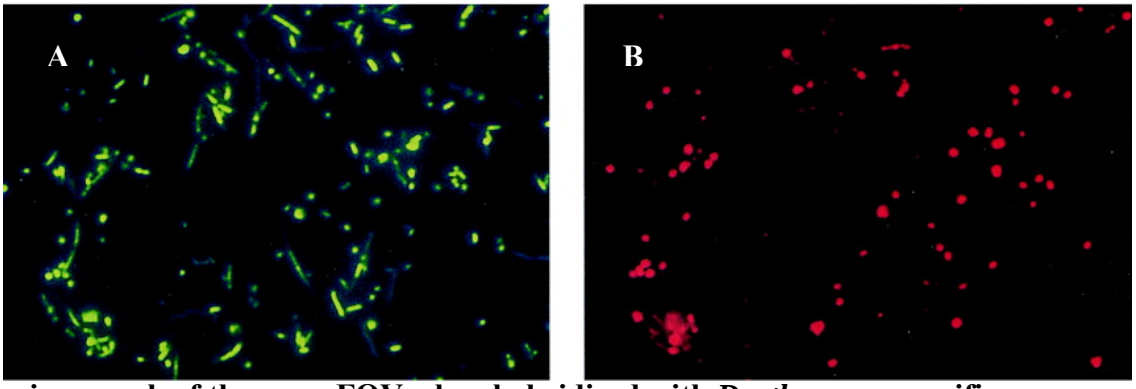
Figure 2.2. Simplified optics of confocal laser scanning microscopy. Source: Ladic, L. http://www.cs.ubc.ca/spider/ladic/confocal.html

(Smith et al., 1994). When applied to microbial aggregates (e.g., biofilms) which require the equipment to resolve spatial resolution at the cellular level, CLSM works better. In CLSM, the confocal pinholes at the laser and at the detector reduce the interference of stray light (Figure 2.2). With the ability to generate a more accurate 2-D image, it can also be used to produce 3-D image by digitizing a sequential series of 2-D (x-y) images obtained from focusing through the specimen in the third (z) dimension (Caldwell et al., 1992).

UV, blue, yellow or red light from the light source is used to energize the specimen on the microscope stage (which may or may not be stained with a

specific stain). The specimen will re-emit light at various wavelengths which then pass out through the eyepieces to be viewed by the observer (Pawley, 1995). For instance, the excitation and emission wavelengths for DAPI are 350 nm and 470 nm, respectively. For fluorochrome CY-3, its excitation and emission wavelengths are 554 nm and 568 nm (Molecular expressions: exploring the optics and microscopy. available at http://micro.magnet.fsu.edu/index.html). Ouverney and Fuhrman (2000) used a UV excitation filter to image the DAPI-stained cells, whereas a green-light excitation filter was used for capturing CY-3 fluorescence. To perform image analysis a digital camera
is used to capture the fluorescent signal. The image then can be exported and image-processing software is used for further analysis.

An excellent tool for accurate and fast identification of microorganisms, FISH has been extensively used in the study of microbial diversity in environmental systems and wastewater treatment applications (sludge), and pathogen studies. FISH so far has been used successfully by researchers to study the microbial communities such as marine habitats (DeLong et al., 1999), a high mountain lake (Alfreider et al., 1996), coastal marine waters (Dang et al., 2002), wadden sea sediments (Llobet-Brossa et al., 1998), marine bacterioplankton (Cottrell and Kirchman, 2000), sites contaminated by chlorinated aliphatic hydrocarbon (Fennell et al., 2001; Richardson et al., 2002), and bulk soils (Zardar et al., 1997). Furthermore, uncultivated species containing known target RNA sequences have also been detected in environmental samples by FISH analysis, such as *Dehalococcoides* (Löeffler et al., 2000; Yang and Zeyer, 2003 [Figure 2.3]), *Methylocella palustris* (Dedysh et al., 2001), *Rhodopseudomonas palustris* (Oda et al., 2000), and iron- and manganese-oxidizing sheathed bacteria (Siering and Ghiorse, 1997). The distribution of microbial types in more complex microbial communities such as wastewater treatment sludge has also been investigated. FISH has allowed the researchers to investigate the presence and spatial distribution of sulfate-



micrograph of the same FOV when hybridized with *D. ethegenes*-specific probes.

reducing bacteria in active sludge (Manz et al., 1998), methanogens in an upflow anaerobic sludge blanket (UASB) (Sekiguchi et al., 1999), and ammonia oxidizing bacteria in a municipal nitrifying activated sludge (Daims et al., 2001). Applicability of FISH analysis has enhanced the medical studies as well. According to the studies conducted by Harmsen et al. (2000), the conventional plate counts of human intestinal flora were approximately ten-fold lower than the corresponding FISH counts. FISH analyses were also applied to other plant and animal pathogen studies (Nour et al., 2000; Loy et al., 1996).

Although FISH can provide significant insight into microbial community structure, there are some factors that can influence its performance and reliability. The precision and reliability of FISH is primarily determined by the specificity of probes. The 16S rRNA may be too well conserved between closely related bacteria to differentiate organisms in the species level. Another problem originates from the fact that there may be a large number of bacteria that haven't been discovered or characterized, and hence no sequence information is available.

A probe that appears to be specific to a particular organism rRNA sequence in a current database may also bind to unknown microorganisms. Similarly, unknown species which are phylogenetic members of a probe target group can be missed due to minor sequence difference (Amann and Ludwig, 2000). Probes should be checked for specificity on a regular basis using the latest sequence databases. Probes may also hybridize with non-target organisms because of noncanonical annealing (e.g., U[T]-U, A-C, A-G, G-U[T]); however, optimal control of the dissociation temperature during the wash stage can minimizing non-specific-binding of probes.

Autofluorescence is another potential problem in the analysis of environmental samples. Moter (2000) found that some organisms autofluorescence such as *Methanogenes* and *Pseudomonas*. Fluorescent chemicals may also be found surrounding the bacteria in environmental samples. However, it has been noted that the growth media and fixiation methods somehow can reduce the autofluorescence interference (Moter 2000). Connally et al. (2002) developed a time-resolved microscopy using fluorophores that can be characterized by long-lived luminescence. The principle for the time-resolved method is that the elapsed time from excitation to capture is set to detect the long-lived emissions. Usually, lifetimes of autofluorescence ranges from 1 to 100 ns whereas commercial fluorophores can last longer than 1.6 ms. Therefore, autofluorescence decays rapidly to low intensity yet the fluorescent dye decays only slightly under the elongated exposure time. Thus, this greatly increases the signal to noise ratio.

Other factors may also obscure the fluorescent signals. The structure of bacterial cell wall, especially for gram positive microorganisms, may hamper probe penetration into cells. Also, the accessibility of 16S rRNA cannot be fully predicted. Loop and hairpin structures and interaction between rRNA and rRNA (and/or rRNA and protein) can reduce the accessibility of the sequence to the probes, and hinder hybridization. The amount of rRNA content in cells varies not only between different species, but also in different physiological stages for a

single cell. It is not surprising to observe abundant rRNA in a physiologically active cell, while levels may be significantly lower in dormant or starving cells.

2.4 Polymerase chain reaction

Polymerase chain reaction (PCR) is an in vitro method for the amplification of DNA. It uses two oligonucleotide primers that are complementary to regions of known sequences (Erlich, 1992). Repetitive cycles involving the denaturation of the duplex template DNA, primer annealing, and extension of the annealed primers by DNA polymerase results in the exponential accumulation of a specific target fragment whose termini are defined by the 5' end of the primers (Giovannoni, 1991).

Except for primers and DNA templates, the components of a PCR reaction mixture can be obtained from many commercial suppliers. A typical PCR reaction mixture contains the following components: DNA polymerase, deoxynucleoside triphosphates (dNTPs), Tris buffer, non-ionic detergent, magnesium chloride, potassium chloride, gelatin or bovine serum albumin (BSA), primers and target DNAs (Taylor, 1991). DNA polymerases carry out the synthesis of a complementary strand of DNA using a single stranded template. The most common DNA polymerase is Taq polymerase which firstly isolated from *T. aquaticus.* It has advantages of heat stability and high temperature optimum. Precursor dNTPs serve as sources of triphosphate, one of the major components of DNA. The salt contents are essential for optimal processivity of the enzyme and amplification.

There are three major steps in PCR (Figure 2.4), which are conducted in an automated cycler which heat and cool the tubes containing reaction mixtures and are usually repeated for 30 or 40 cycles. First, double stranded DNA is denatured to single stranded DNA by briefly heating to 90-95 ºC. The temperature is cooled to 50-55ºC and ionic bonds are formed between the single stranded primer and the single stranded

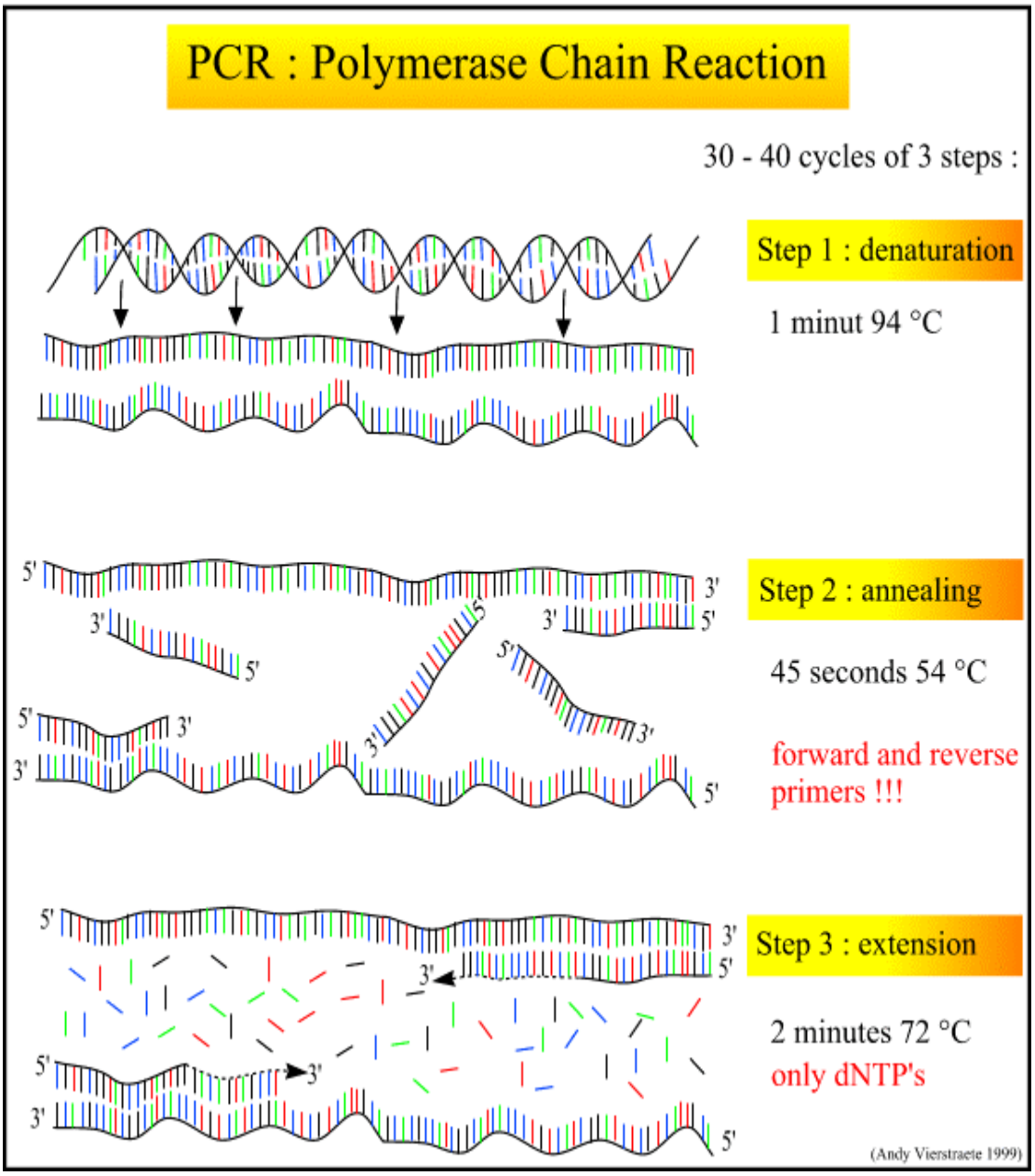


Figure 2.4 Three steps in a typical PCR reaction. Source: Vierstraete, A. http://allserv.rug.ac.be/~avierstr/

DNA template. Annealing the primers stably anneal to the template DNA. The template extension stage is conducted at 70-75 ºC, or the ideal working-temperature range for the *Taq* polymerase used. Bases complementary to the template are covalently joined to the primer on the 3' end. Because both strands are copied during PCR, there is an exponential increase of the number of copies of the sequence bounded by the primer pair (Figure 2.5).

Generally, primers used for PCR reactions are 10 to 20 bases in length. This provides for a practical annealing temperature. The procedure of the primer design and selection in essence is as the same as that of the oligonucleotide probe design. Most importantly, primer pairs should be checked for complementarity. Complementarity between primer sequences at the 3' end often leads to "primer dimmer" formation. The

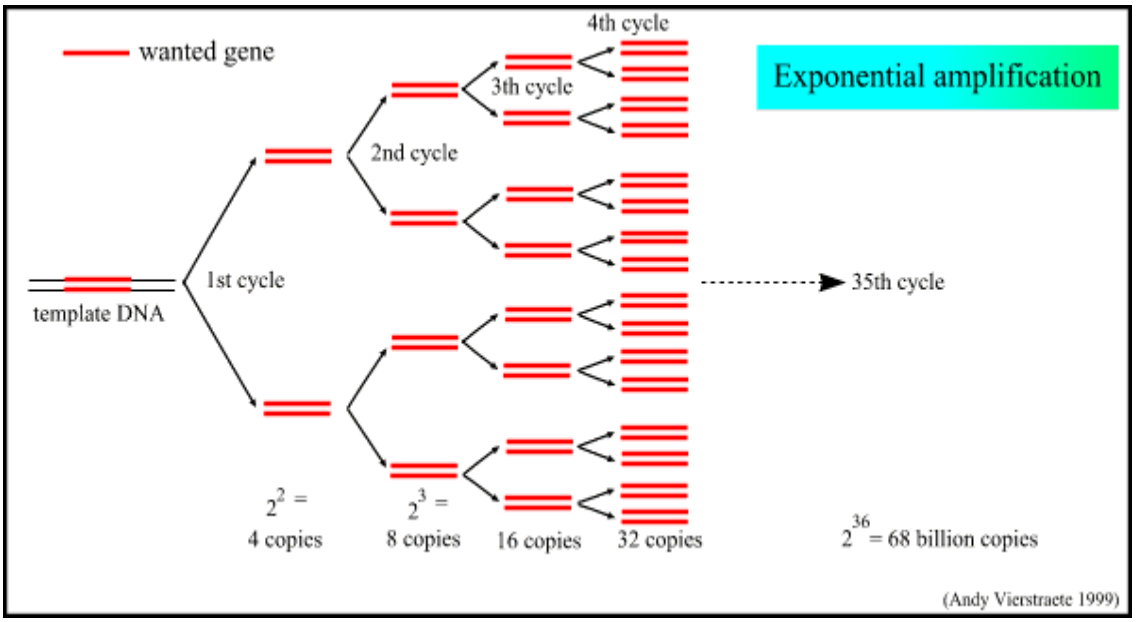


Figure 2.5 The exponential amplification of the gene in PCR. Source: Vierstraete, A. http://allserv.rug.ac.be/~avierstr/

creation and subsequent amplification of these "primer dimmers" reduces the availability of primer to the template molecule resulting in decreased sensitivity or even

failure of the PCR. Furthermore, changes to the PCR reaction buffer (MgCl2, deoxynucleotide triphophates, dNTP, and Taq polymerase) will usually affect the outcome of the amplification (Giovannoni, 1991).

PCR is now one of the most widely used basic molecular biology techniques due to its remarkable speed, specificity, flexibility, and resilience. Hendrickson et al (2002) developed a PCR assay developed to detect *Dehalococcoides* 16S rDNA gene sequences from samples collected from 24 chloroethene-dechlorinating sites in the North America and Europe. PCR has also been used to study the microbial diversity at sites such as hydrocarbon- and chlorinated-solvent-contaminated aquifers undergoing intrinsic bioremediation (Dojka et al, 1998), hot springs in the Yellowstone National Park (Hugenholz et al, 1998; Reysenbach et al, 1994), and gas industry pipelines (Zhu et al, 2003). Fennell et al (2001) used PCR to identify *Dehalococcoides ethenogenes* strain 195 at a TCE-contaminated site. Major et al (2002) were also used the *Dehalococcoides*-specific PCR assay to assess the success of the bioaugmentation of a culture capable of dechlorination of PCE to ethane.

2.5 Real-Time Polymerase Chain Reaction

PCR has been greatly refined since its advent 16 years ago. Several improvements to the technology and related equipment have significantly changed the way PCR is performed today relative to its earlier practice. Of them, the real-time PCR method now allows reproducible quantitation of amplicons at each cycle and, if used with an appropriate standard, allows absolute quantitation of gene products (Heid et al, 1996).

PCR is designed to amplify target sequences in an exponential manner; however, due to inhibitors of the polymerase reaction found with the template,

reagent limitation, or accumulation of pyrophosphate molecules, eventually the PCR reaction no longer amplifies the template at an exponential rate (otherwise known as the "plateau phase"). This is the most important reason why end-point quantification is not very reliable. Real-time PCR permits the detection and measurement of the amount of PCR product during the exponential-amplification stage. It is only during this exponential phase of the PCR reaction that it is possible to extrapolate back to determine the starting amount of template (Ginzinger, 2002). During the exponential phase in the real-time PCR reactions a fluorescent threshold is determined at which point all samples can be compared. It is defined as a function of target signal fluorescence to the amount of background fluorescence and is plotted at a point in which the signal generated from a sample is significantly greater than background. The number of PCR cycles required to generate a strong enough fluorescent signal to reach this threshold is defined as the threshold cycle, or C_T. These C_T values are directly proportional to the amount of starting template.

Currently there are two popular detection chemistries: Taqman probes and SYBR Green I dye. In the Taqman system, three oligonucleotides are used: a forward primer, a reverse primer, and a probe. The probe recognition site resides between the two primers positions and has a quench dye and a reporter dye attached to it. When the probe is intact, the proximity of the quencher greatly reduces the fluorescence emitted by the reporter dye by Förster resonance energy transfer (FRET) through space (PE Biosystems). During the extension stage, the Taq DNA polymerase cleaves the reporter dye from the probe and once the reporter dye separates from the quencher the emitted fluorescent signals can be detected.

SYBR Green I is a double-stranded DNA (dsDNA) binding dye. The dye has very weak fluorescence in free form; but if it binds to dsDNA it emits a strong fluorescent signal. The most important difference between TaqMan and SYBR Green I dye chemistries is that the SYBR Green I dye detects any double-stranded DNA, including non-specific reaction products, where Taqman is specific to amplified sequences containing a recognition site for the Taqman probe. SYBR

Green I can be used to monitor the amplification of any double-stranded DNA sequence without probe requirement, which reduces the assay-setup difficulties and running cost, but may reduce specificity of the detected product. The amplitude of fluorescent signal from SYBR Green Dye I is dependent on the mass or length of the DNA fragment. Hence, under a similar reaction conditions and amplification efficiency, a longer DNA product will generate a stronger signal than a shorter one. However, it may lose specificity since both specific and non-specific PCR dsDNA of PCR products can generate signals in the presence of SYBR Green I. Fortunately, Ririe et al (1997) developed a method that can differentiated amplicons by analyzing the melting curves. The melting curve and the value of T_m are dependent on the G+C content, amplicon length and sequence of the amplicon. For this study, SYBR Green I real-time PCR assay was used for its lower cost and easier sample preparation compared to Taqman, and having 183 BP-specific primers.

There are two methods to calculate the results of the quantitation assays: absolute and relative quantitation. The absolute quantitation method is used to quantitate unknown samples by interpolating their quantities from a standard curve prepared from a pure culture of known cell density. The relative quantitation method is used to analyze changes in gene expression in a given sample relative to reference sample such as an untreated control sample.

The advantages of using real-time PCR over conventional PCR are numerous. For instance, it improves the qualitative PCR assay to allow for quantitation of original template gene copies. Log phase quantification is more accurate and time-saving than the "end-product" quantification. Furthermore, there is no need for "post-PCR" manipulation of sample. Today's real-time PCR instruments even allow performing multiplexing amplification - carrying out two, three or even four PCR reactions simultaneously in a single tube (DeFrancesco, 2003). This development not only increases throughput, but also reduces the cost of reagents and uses less target materials. However, real-time PCR has some

significant limitations. The absolute (standard-curve) quantitation method requires a lot of space on a standard 96-well plate to generate the standard curve. Ginzinger et al. (2002) indicated that sources required for the standard curve may vary, making it difficult to compare data from different plates. In addition, the absolute quantities of the standards must be known by some other independent means, such as direct cell counts or previous purification and quantitation of template gene copies.

Real-time PCR has been widely used in medical research to investigate diphtheria toxin gene (Mothershed et al., 2002) and human and animal pathogens (Edward et al., 2001; Lin et al., 2000; Wang et al., 2002; Aldea et al., 2002; De Medici et al., 2003; Shu et al., 2003). Recently, there has been growing application of this technique in studying environmental microbial phenomena. A real-time PCR assay was developed for rapid and specific detection of *Pfiesteria piscicida* in culture and heterogeneous environmental water samples (Bowers et al., 2000). Using a vinyl chloride (VC)-dechlorinating enrichment culture, He et al. (2003) applied real-time PCR assays to quantify 16S rDNA genes of *Dehalococcoides* catalyzing the critical dechlorination step, and to demonstrate that VC serves as a growth-supporting electron acceptor for the dechlorinating populations. It was found that actively dehclorinating cultures that had dechlorinated 90 μmol of VC contained 51 times more Dehalococcoides gene copies per ml than cultures grown under the same conditions without VC. A TaqMan real-time PCR assay was developed to detect and quantify strain PM1 which degrades methyl tert-butyl ether (MTBE) (Hristova et al., 2001). TaqMan fluorescent signals were directly converted to the measured cell densities in the groundwater samples using the standard curve method, and the results correlated MTBE removal rate to an increase in PM1 population density. Interestingly, Wellinghausen et al. (2001) compared real-time PCR and culture plate counts of detecting *legionellae* from 77 hospital wastewater samples. It was found that the PCR assay not only had a higher detection rate of *legionellae* than the culture method (98.7% over 70.1%), but also

the amounts of *legionellae* calculated from PCR results were higher than the culture results.

2.6 Terminal Restriction Fragment Length Polymorphisms

Terminal restriction fragment length polymorphism (T-RFLP) is a microbial community profiling method based on 16S rDNA gene analysis and can be used with universal primers down to species level primers depending on the information desired. This method, however, does require amplification of the 16S rDNA gene with specific primers and is thus more susceptible to biases and skewing of the native community by amplifying specific gene copies. The technique itself depends on the amplification of template DNA with a primer set, including one fluorescently labeled primer. The resulting amplicons use them subjected to digestion with various restriction endonucleases. The resulting fragments are then analyzed via electrophoresis using an automated sequencer which detects only the fluorescently labeled terminal fragment which is signed based on comparison to internal standards of known size. By this way, individual microorganisms produce a repeatable, specific-sized terminal fragment dependent on the primes and restriction endonuclease used.

Richardson et al (2002) exploited T-RFLP to investigate the microbial community structure in an anaerobic microbial consortium which reductively dechlorinated TCE to ethene. Microbial communities in gluocose-fed methanogenic bioreactor during startup and changes in microbial community structure due to changes in operational parameters were interpreted with T-RFLP (Dollopf et al, 2001). T-RFLP was also used to study the temperature effect on the structure and function of the methanogenic archaeal community in stable cellulose-degrading enrichment cultures (Chin et al, 1999). The T-RFLP method has also been successfully to differentiate four types of soil microbial communities (Dunbar et al, 2000).

2.7 Microbial transformation of 1,1,1-Trichloroethane

1,1,1-Trichloroethane (1,1,1-TCA) is a colorless liquid with a sharp, sweet odor. Its chemical formula is CCl_3CH_3 with a molecular weight of 133.40 g/mol. As a man-made halogenated solvent which is primarily used as an industrial cleaning and degreasing agent, it is one of the most common chlorinated aliphatic hydrocarbons contaminating groundwater. It is slightly soluble in water with a solubility of 1290 mg/L at 25ºC. It can be rapidly transported with groundwater, generating extensive contaminated plumes in the subsurface. With a vapor pressure of 124 mmHg and a Henry's Law constant of 8×10^{-3} atm-m^3/mole at 25ºC, it will volatilize rapidly from water and soil with diffusion through the liquid phase controlling volatilization from water.

The maximum contaminant level (MCL) and maximum contaminant level goal of 1,1,1-TCA in drinking water are 0.2 mg/L (EPA, 2002). It is believed that 1,1,1-TCA has the potential to cause liver, nervous system and circulatory system damage from acute and lifetime exposure at levels above the MCL.

TCA can be reductively dechlorinated through anaerobic pathways to 1,1-dichloroethane (1,1-DCA) by *Desulfobacterium autotrophicum* (Egli et al, 1987) and a *Clostridium sp*. (Galli and McCarty, 1989). Reductive dechlorination of TCA to 1,1-DCA and chloroethane (CA) has been reported in methanogenic consortia (de Best et al 1999) and by a putative *Dehalobacter sp*. (Sun et al., 2002). However, the enzymes that catalyze the reductive dehalogenation of TCA have not been identified.

1,1,1-TCA can also be biotransformed through aerobic cometabolism to 2,2,2-trichloroethanol by the methanotroph *Methylosinus trichosporium* OB3b expressing a soluble methane monooxygenase (Oldenhuis et al., 1989), and by ethane-utilizing *Mycobacterium* spp. isolated from soil (Yagi et al., 1999). Oldenhuis et al (1989) found that *M. trichosporium* expressing soluble methane

monooxygenase completely dechlorinated 1,1-DCA, but degradation intermediates were not identified.

The cometabolism process of 1,1,1-TCA can be carried out by methane-, butane-, and propane-utilizing microorganisms (Rungkamol 2001). Of them, methanotrophs were found not to transform 1,1,1-TCA at a field site at Moffett Airfield, CA (Semprini et al., 1990; Semprini et al, 1991). Kim et al (1997) reported that butane-utilizing microorganisms from Hanford DOE site, WA can degrade 1,1,1-TCA, 1,1-DCE and their abiotic transformation products. A maximum transformation concentration of 1,1,1-TCA of 8310 μg/L by the culture enriched from Hanford, WA From this enriched microcosms, Rungkamol (2001) developed a culture of butane-utilizing microorganics and tested it in groundwater and aquifer solid microcosms that mimicked field conditions at the Moffett field. The microcosms bioaugmented with the culture maintained a long-term (13 days) 1,1,1-TCA transformation in the absence of butane utilization and transforming mixtures of 1,1,1-TCA, TCE ad 1,1-DCE. The inocula has $K_{S,Butane}$ and $K_{S,TCA}$ values of 0.11 mg/L and of 0.37 mg/L, respectively. In a bioaugmented microcosm study, Lim (2003) found that a maximum transformation yield was 0.025 μmol 1,1-DCE/μmol butane and a T-RFL of 183 base pair was dominant in the bioaugmented microcosm fed butane and simultaneously fed butane and 1,1-DCE.

2.8 Bioaugmentation

By definition, bioaugmentation is a bioremediation strategy that enhances remediation of an immediate release of pollutants by addition of an active biomass, or augments the capabilities of indigenous microbial community that is incapable of degrading existing pollutants (Baud-Grasset and Vogel, 1995). This technology is applied when the indigenous microorganisms are unable to degrade the target compounds because of absence of appropriate enzymes, insufficient population of microorganisms (biomass), or to counteract compound toxicity (Rungkamol, 2001). With bioaugmentation, a sufficient amount of specifically

acclimated microorganisms are applied to the contaminant treatment zone leading to higher biodegradation levels.

Vogel (1996) listed some of the factors crucial to the success of bioaugmentation including contaminant characteristics (bioavailability, concentration, and microbial toxicity), soil physical and chemical properties (soil type, organic matter content, humidity, and pH), microbiology (enzyme activity and stability) and methodology (method of inoculation). However, a major concern of bioaugmentation is the survival of the inoculated microorganisms. Martin (1994) indicated that nutrient limitations (P, N, and other possible elements), suppression by predation and parasites, inability of bacteria to move appreciably through soil, concentration of organic substrate too low to support multiplication, improper pH, temperature and salinity, and toxins can cause bioaugmentation to fail.

During the bioaugmentation process, microbial sampling is performed as well as hydrological and chemical sampling to evaluate treatment effectiveness. Salanitro et al. (2000) routinely monitored MTBE degrading microbes in groundwater samples using the extinction dilution (most probable number, MPN) method during a bioaugmentation study at the USN Hydrocarbon National Environmental Test site at Port Hueneme, CA. Major et al. (2002) conducted a field demonstration to evaluate bioaugmentation of a dechlorinating enrichment culture that contained phylogenetic relatives of *Dehalococcoides ethenogenes* to dechlorinate PCE to ethene. Throughout the study, groundwater samples were monitored for the presence of Dehalococcoides using a *Dehalococcoides*-specific PCR assay. They found that sequences identical to that of the inoculated *Dehalococcoides sp*. culture were detected after bioaugmentation. Combined with gel electrophoresis image analysis, it was observed that there was a growing population of inoculated culture with distinct spatial distribution within the bioaugmented aquifer, which correlated to the observed dechlorination of chlorinated ethenes. In a treatment study of metal-working fluids in a bioaugmented bioreactor, 16S rDNA amplification, denaturing gradient gel

electrophoresis (DGGE) and FISH were conducted to evaluate the inocula performance (van der Gast et al., 2003). FISH data revealed that the inocula were highly competitive over the indigenous microbial community and well-adapted to the toxic reactor environment 100 hours after bioaugmentation. The dominance of the augmented microorganisms was also confirmed by community analysis with PCR and DGGE.

CHAPTER 3
MATERIALS AND METHODS

In the present study, microbial DNA was extracted from three types of samples: aqueous media culture samples from media growth bottles in the laboratory; groundwater samples from the bioaugmentation test in the Moffett Field and soil column effluent samples; and, solid samples obtained from solid coupons obtained in the field. Microbial community DNA was performed with T-RFLP analysis to investigate the bacterial community structure and dynamics corresponding to 1,1,1-TCA biodegradation. Patterns of T-RFLP profiles were performed with similarity analysis. Two primers were designed specific to strain 183BP. In order to quantify strain 183BP populations in the groundwater and soil column effluent, a real-time SYBR Green PCR method was developed. A general method of FISH analysis was also described in this chapter though no significant fluorescence of FISH-labeled cells was observed. As a result, all the cell quantitation in this study was based upon real-time PCR analysis.

3.1 Preparation of DNA templates

Three different cell sample preparations were used dependent upon the sample types. All the concentrated cells then proceeded to DNA extraction using the same protocol described in 3.1.4.

3.1.1 Aqueous media cultures

Sampling of cultures grown in the laboratory consisted of 0.1 to 1 ml of cell culture (dependent on the cell density) was pipetted into a clean 1.5 ml microcentrifuge tube (Eppendorf, Germany) and centrifuged at 9000×*g* for 3 min. Supernatant was removed and the pellet was incubated in lysozyme buffer to begin the DNA extraction procedure.

3.1.2 Groundwater and soil column effluent

Groundwater samples were collected in 250 ml polycarbonate bottles (Nalgene, Rochester, NY) from sampling wells at the Moffett bioaugmentation test site, CA and shipped in ice chests to Oregon State University. Cells were then concentrated from groundwater samples by filtration through 0.2μm-pore-size white polycarbonate filter membranes (diameter, 25 mm; Osmonic Inc.) with 0.8μm-pore-size nitrocellulose support membrane (diameter, 25 mm; Millipore) by applying a vacuum of 20 in. Hg. The polycarbonate filter membrane was then placed in a clean 1.5 ml microcentrifuge tube (Eppendorf, Germany) and incubated in lysozyme buffer to begin the DNA extraction procedure.

Occasionally the presence of small soil and/or organic particles contained in the groundwater samples caused very serious clogging of the membrane during filtration. When filter clogging caused excessive filtration times, a centrifuge method for all concentration was applied. A 240-ml portion of the water sample was equally dispensed into 6×50 ml centrifuge tubes (VWR International, San Diego, CA) and centrifuged at 5000×*g* for 10 min. The supernatant from each tube was removed and the final 1.5 ml was transferred to a clean 1.5 ml microcentrifuge tube (Eppendorf, Germany) and centrifuged at 9000×*g* for 3 min. Approximately 200 μl resulting sediments was taken from the bottom of each tube and combined in a new 1.5 ml tube and again centrifuged at 9000×*g* for 3 min. All the resulting sediments were placed into a new 1.5 ml

microcentrifuge tube (Eppendorf, Germany). After the final centrifuge step, supernatant was removed and the sample was incubated in lysozyme buffer to begin the DNA extraction procedure.

Approximately 250 ml of effluent samples from the soil column were collected twice a week. The cells of the aqueous samples were concentrated using the same method as the groundwater samples.

3.1.3 DNA extraction

DNA extraction from samples obtained from the field site, the soil column and the cell cultures was performed by following the protocol supplied with the DNeasy Tissue Kit (Qiagen, Valencia, CA). Briefly, concentrated cell pellets or the filter membrane containing cells were suspended in a cell lysis buffer (200 µl per tube) containing 20 mM Tris-Cl (pH 8.0), 2 mM EDTA, 1.2% Trition X-100 and 20 mg/ml lysozyme. After incubation at 37 ºC for 30 min, 25 µl proteinase K solution and 200 µl buffer AL were added the cell solution and heated to 70 ºC for another 30 min. Then, 200 µl of 100% ethanol was added to deproteinate the lysates. The mixture was pipetted into a DNeasy spin column placed in a 2 ml collection tube and centrifuged at 8000×*g* for 1 min. The spin column was transferred to a new collection tube, 500 µl buffer AW1 was added, and the tube was centrifuged at 8000×*g* for 1 min. Then the spin column was placed on a new collection tube and 500 µl buffer AW2 was added to center of the spin column. Then the tube was centrifuged at 14000×*g* for 3 min. After discarding the flow-through, the mixture was centrifuged at the same speed for 1 more min to allow complete ethanol evaporation/removal. The spin column was then transferred to a clean 1.5 ml microcentrifuge tube (Eppendorf, Germany) and DNA was eluted with 100 µl of elution buffer AE and stored at -20ºC.

3.2 Design of oligonucleotide probes and primers

The goal of probe (primer) design was to choose an ideal oligonucleotide sequence complementary to a region of the target sequence that allows at least one mismatch to the same region in all other non-target organisms. Since the primer design follows the same procedure as probe design, only the probe design procedure is described here. Potential 16S rRNA-targeted oligonucleotide probes applicable to the specific detection of strain 183BP were formulated using the probe design tool of the ARB program package (written by O. Strunk and W. Ludwig; available at http://www.arb-home.de). Operating under UNIX, LINUX or Sun-Micro system, it is a graphically oriented software package for phylogeny analysis, sequence data analysis and molecular probe design.

Figure 3.1 shows the procedure for probe design. Fragment sequences of strain 183BP were compiled using the ARB CONSENSUS function. To providing sufficient information of non-target, but phylogenetically similar sequences to strain 183BP, the partial sequence of strain 183BP (the first 1032 bases) was checked against the Genebank database using BLAST at NCBI (http://ncbi.nlm.nih.gov/BLAST). Fifty sequences sharing the highest similarities with strain 183BP were obtained from NCBI and imported into the ARB program along with the strain 183BP sequence as well. These sequences were added to a 16S rRNA database downloaded from RDP II (Hugenholtz, 2002; available online at http://rdp.cme.msu.edu/html/alignments.html), which contains more than 8000 sequences, most belonging to soil bacteria. The imported sequences were automatically aligned using FAST ALINGER (version 1.03) against the existing sequences. The aligner needs a sequence as reference, which can be chosen from the consensus of the group containing the species, from the next relative found by the selected PT_SERVER, or from a fixed species if no relative is found automatically. Ambiguously and incorrectly aligned positioned were manually aligned on the basis of the conserved primary sequences and consensus of phylogenetically related sequences.

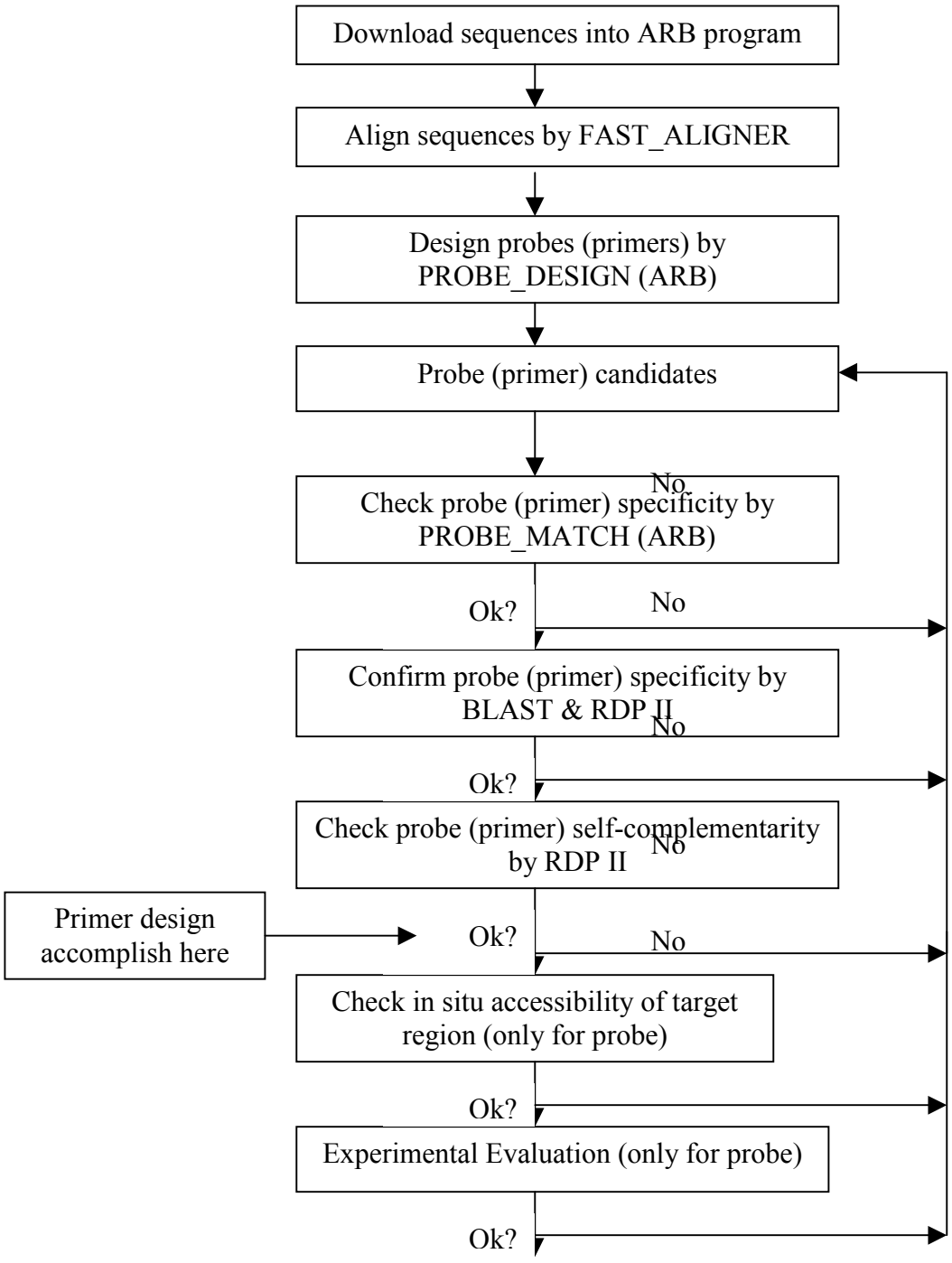


Figure 3.1 Flowchart of probe (primer) design and selection

Based on the newly established 16S rRNA database, the probe design tool can select nucleotide sequence regions that allow discrimination of the target sequence from all non-target reference sequences. In the ARB program, PT_SERVER searches for patterns (such as regions specific to target sequences) in special searchable database files, which essentially are fragmented versions of standard ARB database files (Hugenholtz et al., 2001). Before performing the probe design, the searchable database file, PT_SERVER, must be updated from the newly modified ARB database. The 183BP sequence was highlighted as the design target while others were left unmarked. Several parameters need to be defined prior to probe design, such as G+C content, location (E. coli number), maximal hairpin bonds, melting temperature and probe length. In this case, they were defined as 50 to 100%, 1 to 10000, 0, 30 to 100 ˚C, and 18-mer, respectively. Potential probe (primer) candidates were evaluated by the PROBE_MATCH function in order to check their specificity from no mismatching up to 5 mismatches. The criterion for the probe selection is that the probe should contain at least one mismatch to non-target sequences, ideally located in the middle of the target string to maximize the destabilizing effect of the mismatch.

Once a probe (primer) was characterized that had at least one mismatch to all non-target sequences in the database, probe specificity was checked against all available rRNA sequences in the public databases (Genebank and RDP II [available at http://rdp.cme.msu.edu/html/]). An exact match will have a score (bits) of twice the value of the number of nucleotides of the submitted probe sequence (Table 3.1 and Table 3.2).

The hybridization and PCR reaction can be disrupted if the probe is self-complementary, i.e., forming hairpin turns or dimers. Thus, the probe sequence should be inspected by the PROBE_MATCH program in RDP II, where only pairings of 2 or more consecutive Watson-Crick pairs are accepted and the allowed size of the loop (of the potential hairpin) is between 2 and 4.

The accessibility of the probes (primer) was further evaluated by comparing the 16S rRNA target position of the probes with the in situ accessibility map of E. coli provided by Fuchs et al. (1998). They examined the accessibility of the E. coli rRNA to more than 200 oligonucleotide probes complementary to the entire length of the E. coli 16S rRNA molecule and mapped the regions with relatively high and low accessibility. However, this check provides only a very general idea about the putative fluorescence intensity of the individual probe because the secondary structure of E. coli 16S rRNA may differ from that of other bacteria strains, especially for bacteria strains phylogenetically distant to *E. coli*.

Table 3.1 Bacterial sequences that are complementary to the primer Ran191F

Genebank Accession #	Bacterial sequences producing significant alignments	Score (bits)	E value
X80625	*Rhodococcus rubber* strain DSM43338T	36	0.27
AY247275	*Rhodococcus ruber* M2	36	0.27
AF529079	*Rhodococcus sp*. an 16S rRNA gene	36	0.27
AY114177	*Rhodococcus ruber* strain AS 4.1038	36	0.27
AF420413	*Rhodococcus sp*. USA-AN012	36	0.27
AY114109	*Rhodococcus sp*. E33	36	0.27
AF447392	*Rhodococcus aetherovorans* strain Bc663	36	0.27
AF447391	*Rhodococcus aetherovorans* strain 10bc312	36	0.27
AF323255	Gram positive bacterium B1G-1B	36	0.27
AF131484	*Rhodococcus sp*. IM-43760	36	0.27
AF103733	*Rhodococcus sp*. YH1	36	0.27
AF350248	*Rhodococcus ruber* strain AS4.1187	36	0.27
AJ459106	*Rhodococcus sp*. HN	36	0.27
AY496284	*Rhodococcus sp*. SoD	36	0.27

Table 3.2 Bacterial sequences that are complementary to the primer Ran443R

Genebank Accession #	Bacterial sequences producing significant alignments	Score (bits)	E value
AF547972	*Mycobacterium tokaiense* strain CIP 106807	36	0.27
AF547950	*Mycobacterium murale* strain CIP 105980	36	0.27
AF420413	*Rhodococcus* sp. USA-AN012	36	0.27
AF447392	*Rhodococcus aetherovorans* strain Bc663	36	0.27
AF447391	*Rhodococcus aetherovorans* strain 10bc312	36	0.27
AF480590	a *Mycobacterium tokaiense* 16S rRNA gene	36	0.27
Y08857	a *Mycobacterium sp.* 16S rRNA gene	36	0.27
AB009578	*Mycobacterium sp*. PP1	36	0.27
AF353688	*Rhodococcus sp*. PR-N14	36	0.27
AF103733	*Rhodococcus sp*. YH1	36	0.27
AF350248	*Rhodococcus ruber* strain AS4.1187	36	0.27
AJ459106	*Rhodococcus* sp. HN	36	0.27

3.3 PCR

3.3.1 PCR Assay

The 16S rDNA gene sequences were amplified by using "183BP-specific" primers or the universal bacteria primers shown in Table 3.3.

Table 3.3 16S rDNA primer sequences and concentrations in PCR reaction mixtures

Primer	Primer Sequence	Concentration (pM)
Universal bacteria primers		
27F-B-FAM*	5'-AGR GTT YGA TYM TGG CTC AG-3'	40
338Rpl	5'-GCW GCC WCC CGT AGG WGT-3'	30
183BP-specific primers		
Ran191F	5'-GTT CCG GGG TGG AAA GGT-3'	20
Ran443R	5'-ACT CGC GCT TCG TCG GTA-3'	20

*27F-B-FAM primer is labeled with FAM dye at the 5' end for T-RFLP analysis
**All primers are purchased from Qiagen Inc., Valencia, CA.

Each PCR reaction was carried out in a final volume of 50 µl. Reaction mixture contained 20 ul 2.5×PCR Eppendorf MasterMix (Eppendorf, Westbury, NY) giving final concentrations in the PCR reaction of 1.25U Taq DNA

polymerase, 50 mM KCl, 30 mM Tris-HCl, 1.5 mM Mg2+, 0.1% lgepal-CA630 and 200 µM each dATP, dCTP, dGTP and dTTP, 20 – 40 pmol of each primer (a forward primer and a reverse
primer, Table 3.3), and 2 µl of template DNA. All PCR amplifications were performed with an Eppendorf Gradient Thermocycler (Eppendorf, Germany). The PCR reaction conditions consisted of one cycle of 95 ºC for 2.5 min, 55 ºC for 1 min, and 72 ºC for 2 min, followed by 35 cycles of 95 ºC for 0.5 min, 55 ºC for 1 min, and 72 ºC for 2 min, and final extension at 72 ºC for 20 min and cooling at 4ºC. The annealing temperature (55 ºC) was the same for all primers used.

Molecular biology grade water was prepared by filtering nanopure water (NANO pure, Barnstesd International Inc., Boston, MA) through a 0.2 µm syringe filter (Millipore Corp., Billerica, MA), followed by UV-light exposure for 1 min. Molecular biology grade water (2 µl) and DNA template extracted from a pure strain 183BP culture were used as negative and positive controls, respectively, in PCR reactions.

3.3.2 Gel Electrophoresis

Five microliters of each PCR product was visualized on a 1% agarose gel (EMD Chemicals Inc., Gibbstown, NJ) prepared with 1×TAE buffer (0.04M Tris-acetate, 0.001M EDTA) and stained with 0.5 µg/ml ethidium bromide. MassRuler Low Range DNA Ladder (MBI Fermentas Inc, Hanover, MD) was loaded as a DNA mass marker. The DNA Ladder yields the following 11 discrete fragments (in base pairs): 1031, 900, 800, 700, 600, 500, 400, 300, 200, 100, and 80. Electrophoresis of 5µl MassRuler DNA Ladder results in bands containing 50, 45, 40, 35, 30, 50, 20, 15, 10, 5, 4 ng of DNA, respectively. Electrophoresis was conducted using 1×TAE at 220 volts for 40 min.

The resultant gels were imaged by BioDoc-It system (UVP Inc, Upland, CA) and the DNA band intensities in the electronic images were analyzed using the NIH Image J program (written by Wayne Rasband of the National Institute of

Health; available at http://rsb.info.nih.gov/ij/ for free). Band intensities and sample volumes were used to estimated the required dilution to prepare samples for fragment analysis at Central Service Lab at Oregon State University

3.4 T-RFLP

PCR products amplified by the primer pair of 27F-B-FAM and 338R were digested at 37 ºC for 4.5 hours with *Mnl*I or *Hin6*I restriction endonucleases (MBI Fermentas Inc, Hanover, MD). Digestion was followed by incubation at 80ºC for 10 min to terminate enzyme activity. Each enzyme was supplied in a concentration of 10 U/µl with an appropriate buffer. The recognition sites of these enzymes and the composition of the reaction mixtures are shown in the Table 3.4.

One microliter of each digested sample was taken and diluted to an approximate concentration of 1 ng DNA/µl with molecular biology degree water (The dilution ratio was calculated from DNA mass estimation by Image J). One microliter of the diluted DNA sample was then taken and dried using a Vacufuge concentrator (Eppendorf, Germany) under aqueous mode for 20 min. The dried pellet was sent to the Central Service Lab at Oregon State University for fragment analysis.

Table 3.4 Enzyme recognition sites and reaction components for restriction enzyme digestions

MnlI		Hin6I	
Recognition sites			
5'-C C T C$(N)_7$ ^-3' 3'-G G A G$(N)_6$ ^-5'		5'-G^C G C-3' 3'-C G C^G-5'	
Reaction mixture ingredients			
Reagent	Volume (µl)	Reagent	Volume (µl)
Enzyme (10 U/µl)	0.25	Enzyme (10 U/µl)	0.25
G^+ Buffer	2	Y^+ / Tango Buffer	2
PCR product	10	PCR product	10
Water	7.75	Water	7.75

Each sample was run on an ABI 377 slab gel platform with Filter set A as the dye set consisted of 6-FAM (blue) labeled samples and Rox (red) standard. GENESCAN400-ROX (fragment size: 50-, 60-, 90-, 100-, 120-, 150-, 160-, 180-, 200-, 220-, 240-, 260-, 280-, 290-, 300-, 320-, 340-, 360-, 380- and 400-bp; PE Applied Biosystems, Foster City, CA) was added into each sample as an internal lane standard. Fragment size was determined using GeneScan software, version 2.1 (PE Applied Biosystems, Foster City, CA). Results were obtained in the form of an electropherogram and a data table of fragment size and peak area.

The densitometric curve of each T-RFLP file was transferred to GelComparII program (version 3.5, Applied Maths Inc., Belgium). Each gel was normalized according to the internal size standard and the banding patterns were compared using unweighted pair group method with arithmetic means clustering method and the Dice coefficient method.

3.5. Real-time PCR

3.5.1 Optimization of primer concentration

Primer concentrations to be used were determined by varying the concentration of forward and reverse primers, so the concentrations that provide optimal assay performance could be determined. The primer concentrations selected should provide a low C_T and high fluorescence signals when run against the target template, but should minimize non-specific product formation in the non-template controls. In this case, primer titration were performed using 50/50, 150/150, 300/300 nM concentrations of forward and reverse 183BP-specific primers, Ran191F and Ran443R. The optimal concentration was found to be 150 nM.

3.5.2 SYBR Green I quantitative PCR

The SYBR Green real-time PCR was performed in a Sequence Detection System 7000 (PE Applied Biosystems, Foster City, CA). The SYBR Green PCR Master Mix (PE Applied Biosystems, Foster City, CA) contains SYBR Green I Dye, AmpliTaq Gold® DNA polymerase, dNTPs with dUTP, Passive Reference 1, and optimized buffer components. All amplification reactions were carried out in a total volume of 25 µl in 96-Well Optical Reaction Plates and optical adhesive covers (PE Applied Biosystems, Foster City, CA). Each reaction contained 1 µl of DNA template, 12.5 µl of 2×SYBR Green I PCR Master Mix, 150 nM concentrations of forward and reverse primers, and, to reach a total volume of 25 µl, molecular biology grade water. The cycling parameters were as follows: initial incubation at 50ºC for 2 minutes to active UNG and for 95ºC for 10 minutes to activate the Taq polymerase, thermal conditions followed 40 cycles of 95ºC for 15 seconds and 60ºC for 1 minute, during which a fluorescent measurement was taken from each well at each cycle.

For this study, an absolute quantitation method was used. Calibration curves were run in parallel and in triplicate with each analysis using the DNA extracted from a pure strain 183BP culture. The extracted DNA was then diluted in 10-fold series from 4.04×10^{-1} down to 4.04×10^{-6} per ml. Wells with no DNA template were used as negative controls. The cycle threshold (C_T), indicative of the quantity of target gene at which fluorescence exceeds a preset threshold, was set above the baseline where there was little change in the fluorescence signal. After exceeding the threshold, the sample was considered positive.

Amplification data collected by the 7000 Sequence Detector was then analyzed using the Sequence Detection System software (version 1.1; PE Applied Biosystems, Foster City, CA). The calibration curve was created by plotting the cycle threshold (C_T) against the log of the concentrations of strain 183BP. The coefficient of linear regression (R) for each standard curve was calculated.

10-fold serial dilutions of DNA extracted from a pure culture of strain 183BP containing [4.04 ± 2.18] $\times 10^7$ cell/ml were prepared. Standard curves were performed in triplicates. Initially PCR was performed on triplicates of serial dilution in the same run, 1:1, 1:10 and 1:100. However, the C_T values of 1:1 and 1:10 dilution were very close. We believed that the presence of humic or other background compounds in the environmental samples inhibited the PCR reaction. A clear 1-log decrease in the sensitivity of 183BP detection was observed between 1:10 and 1:100 dilutions for most samples. Due to the high variability associated with 1:100 diluted samples, samples of 1:10 dilution were chosen for cell quantitation.

3.5.3 Amplicon specificity analysis

SYBR Green I is a double-stranded-DNA-binding DNA. When in free form, it has no detectable fluorescence; but, it can emit fluorescence when binding to any double-stranded (ds) DNA (Giulietti et al., 2001). Following amplification, a melting curve analysis was performed to verify the product specificity by its melting temperature (T_m) (Ririe et al, 1997). If SYBR-Green-I-bound dsDNA is heated up, a rapid loss of SYBR Green I fluorescence can be observed near the denaturation temperature. The melting curve and the value of T_m are dependent on the GC content, length and sequence of the amplicon. Thus, the amplicons can be distinguished by the melting curve analysis. In the present study, T_m is determined during a phase of slow heating, from 60 to 95ºC, during which time there occurs a rapid decrease in the fluorescence due to the denaturation of the amplicons, resulting in the formation of single filaments of DNA and the successive detachment of the SYBR Green I. The melting curves were visualized with the Sequence Detection System software (version 1.1; PE Applied Biosystems, Foster City, CA). Curves with more than one evident melting point were not used for analysis.

3.6 FISH

3.6.1 Oligonucleotide probes and stains

Once selected, the oligonucleotide probes were purchased (Operon Technologies, Inc., Alameda, CA) with a single amino linker attached at the 5' terminus and labeled with cyanine Cy3 monofunctional dye. The probe pellets were dispensed to 0.5 ml amber safe-lock microcentrifuge tubes (Eppendorf, Germany) with 170 pmol per tube were dried and stored at –20 °C. Prior to use, the probe stock solution was diluted to a final probe concentration of 2 ng/µl using hybridization solution (0.9 M NaCl, 20 mM Tris-HCl [pH 7.4], 0.01% sodium dodecyl sulfate, and 15% or 35% formamide).

The DNA intercalating dye 4', 6'-diamidino-2'-phenylindole (DAPI; Sigma) was stored as a solution at 1 mg/ml at –20 °C. A final concentration of 5 µg/ml in hybridization wash solution (150 mM NaCl, 20 mM Tris/HCl [pH 7.4], 5 mM EDTA, and 0.01% sodium dodecyl sulfate) was placed in coplin jars at 4 °C and used to nonspecifically stain bacterial cells and any other DNA-containing organisms.

3.6.2 Fixation and filtration

Groundwater samples were fixed in final concentration of 2% Paraformaldehyde solution for 4 hours at 4°C. After fixation, 20 ml individual sub-samples were filtered through 0.2 µm pore size white polycarbonate filter membrane (diameter, 25 mm; Osmonic Inc.) with 0.8 µm pore size nitrocellulose membrane support (diameter, 25 mm; Millipore Inc.) and glass microfibre filter backing (diameter, 25 mm; Whatman) by applying a slight vacuum at ~5 in. Hg. After filtration, the polycarbonate membrane was transferred from filter stack to a microscope slide (size, 25×75×1 mm; Fisher Scientific) using Cryo-Babies

(Diversified Biotech), and air-dried in the dark, and stored at –20 °C in a slide box containing desiccants.

3.6.3 Whole-cell hybridization and DAPI staining

Prior to hybridization, the white polycarbonate membrane was cut into four parts and each quarter was fixed to a new slide with a Cryo-Babies (Hampton Research, Aliso Viejo, CA) on each end. Hybridization with oligonucleotide probes for each quarter membrane was performed in a 50 µl aliquot of hybridization solution (0.9 M NaCl, 20 mM Tris/HCl [pH 7.4], 0.01% sodium dodecyl sulfate, and 15% or 35% formamide, 2 ng/µl probe) pipetted directly onto the membrance. A glass cover slip (size, 24×60 mm or 22×22 mm; VWR International, San Diego, CA) was applied immediately on the top of the Cyro-Babies in order to create a layer of hybridization solution between the cover slip and the membrane. The slide was then placed in a sealed black chamber containing 3 small jars filled with nanopure water, and incubated at 37 °C for 3 hours for media-grown cultures and 10-16 for environmental samples. Following hybridization, the slide was transferred to hybridization wash buffer (150 mM NaCl, 20 mM Tris/HCl [pH 7.4], 5 mM EDTA, and 0.01% sodium dodecyl sulfate) for 10 minutes. The same wash step was repeated once in a new hybridization wash buffer. The slide was then transferred into the 5 µg/ml DAPI solution and incubated for 10 minutes in the dark and then rinsed with DAPI wash (hybridization wash) for another 2 minutes, and finally air dried in the dark with the Cryo-Babies stickers removed. A drop of Citifluor antifadent (Ted Pella, Inc., CA) was added to each membrane and then covered with a cover slip, sealed with nail polish, and stored at –20 °C with desiccant.

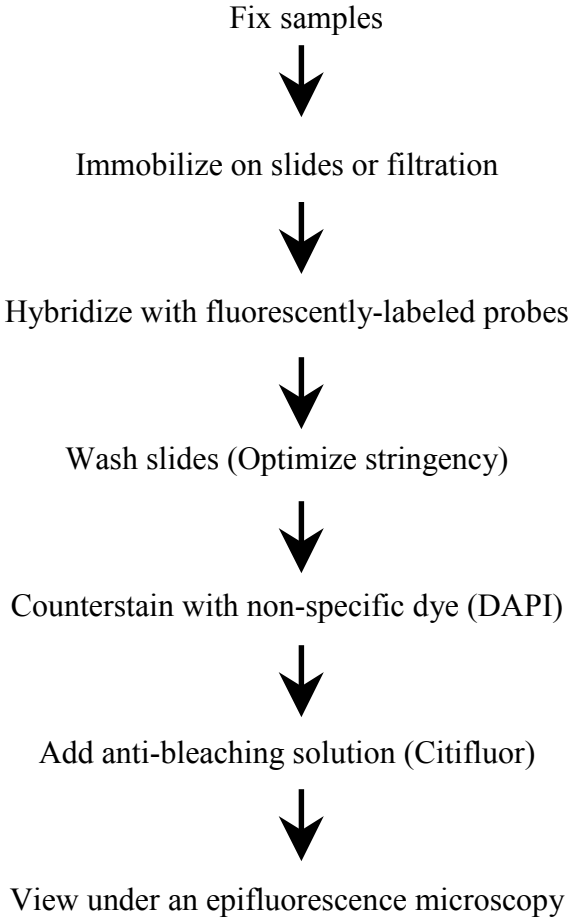


Figure 3.2 Common FISH procedure

3.6.4 Cell counts

Total cell counts and FISH-labeled cell counts were calculated by the method developed from Kepner and Pratt (1994). Cell counting was performed on randomly chosen field of view (FOV) for each test sample. The number of target cells per milliliter of groundwater sample was determined from values for the effective area of the filter, the area of FOV, the dilution of the sample applied, and the volume of diluted sample filtered, as follows:

$$\text{Total number of target (cell/ml)} = (N \times A_t)/(d \times V_f \times A_g)$$

Where, N: mean number of cell counted per FOV;
A_t: effective area of the filter (mm^2)
d : dilution factor (V_{final}/V_{sample})
V_f : volume of diluted sample filtered (ml)
A_g: area of FOV (mm^2)

3.6.5 Fluorescent microscopy

Cy3-positive and DAPI-positive cells were counted for each field of view using a DMRB epifluorescence microscope (Leica Inc, Germany) coupled with an ORCA-ER CCD digital camera (Hamamatsu Inc., Japan) and filter sets appropriate for Cy3 and DAPI. Consistent exposure times for DAPI and Cy3 images were 1 and 5 seconds, respectively. Cy3 and DAPI images were processed by IPLab software (version 3.5.5, Scanalytics Inc, Fairfax, VA). Positive probe signals were determined by segmenting and overlaying Cy3 images on the corresponding DAPI image (the same FOV under the DAPI filter set) segmentations. Segmentations on the Cy3 images matching the corresponding segmentations on the DAPI images were counted as positive. Negative control counts were determined using the same technique and subtracted from positive probe counts to correct for autofluorescence and nonspecific binding.

CHAPTER 4
MOFFETT BIOAUGMENTATION TEST (OCTOBER, 2003)

4.1 Introduction

Groundwater contamination is of significant health concern since it is used as a drinking water supply by many people. 1,1,1-Trichloroethane (1,1,1-TCA) is a man-made halogenated solvent and has been primarily used as an industrial cleaning and degreasing agent. It is one of the most common CAH contaminants found in groundwater and soil environments. The maximum contaminant level (MCL) and maximum contaminant level goal (MCLG) of 1,1,1-TCA in drinking water are 0.2 mg/L (EPA, 2002). Bacterial cultures isolated from various environmental media are capable of degrading 1,1,1-TCA either through aerobic cometabolic or anaerobic pathways (Egli et al, 1987; Galli and McCarty, 1989; de Best et al 1999; Yagi et al., 1999; Kim et al, 1997).

The Moffett Federal Airfield In-Situ Bioremediation Test Site (Mountain View, CA), where various bioremediation filed tests have been conducted, is a site contaminated with various chlorinated aliphatic hydrocarbons (CAH), including 1,1,1-TCA. Remediation assessment studies conducted at Moffett field have shown that bacteria that use methane as primary substrate and cometabolically transform CAHs with a methane monooxygenase enzyme achieved significant transformation of vinyl chloride (VC) and trans-1,2-dichlroethene (t-DCE), but exhibited limited transformation of 1,1,1-TCA, trichloroethylene (TCE) and cis-1,2-dichlroethene (c-DCE) (Semprini et al., 1990; Semprini et al., 1991). In other field tests (Hopkins et al., 1993; Hopkins and McCarty, 1995), phenol- and toluene-oxidizing bacteria showed equally effective removal of c-DCE and TCE, but t-DCE was not as effectively transformed. Fries et al (1997) identified 63 strains from 273 phenol- and toluene-degrading isolates cultured from cotton-fiber roughing filters used to remove sediments from the recirculated groundwater

during operation of the Moffett field test site. Some of the isolates were capable of TCE oxidation and some were not, indicating significant microbial diversity and redundant and differential functionality at the site. In microcosm studies conducted with aquifer material obtained from the Moffett Field site, none of the microcosms effectively degraded 1,1,1-TCA when fed phenol, toluene, methane or ammonia (Hopkins et al, 1993; Hopkins and McCarty, 1995).

Several bioaugmentation treatment tests conducted at contaminated sites have resulted in successful transformation of the targeted toxins to harmless compounds (Baud-Grasset et al, 1995; Fantroussi et al, 1999; Major et al, 2002; Salanitor et al, 2000). Bioaugmenting microorganisms with known transformation abilities may improve biotransformation of chlorinated aliphatics at Moffett Field. Kim et al (1997) enriched a mixed community from a CAH-contaminated DOE site in Hanford, WA. The enrichment culture was able to cometabolize 1,1,1-TCA, 1,1-DCE and a number of other CAHs using butane as a primary substrate. A butane-utilizing organism, described here as strain 183BP, was isolated from the enrichment and was shown to have 1,1,1-TCA, 1,1-DCA, and 1,1-DCE transformation abilities (Mathias, 2001). Characterization of a 1-kb fragment of the 16S rRNA gene of strain 183BP revealed 100% identity to a known *Rhodococcus sp*. USAN-12 (Genbank accession number AF420413). During the past few years, a number of field bioaugmentation pilot tests using cultures containing strain 183BP have been conducted at the Moffett field test site. The ability of the inocula to survive, adapt and flourish in a non-native environment and function well in transforming the targeted contaminants is an important concern in bioaugmentation.

Microbial analysis may provide a critical key in understanding the contaminant biodegradation process. Traditional approaches like culture plating and the most probable number (MPN) method are often too selective, time-consuming and inaccurate. The development of molecular-based techniques allows detecting and/or quantifying microorganisms in different environments using 16S

rDNA/rRNA genes (Hugenholz et al, 1998; Hendrickson et al 2002; Dojka et al, 1998; Fennell et al 2001; Major et al, 2002; Löeffler et al., 2000; DeLong et al., 1999).

To date, bioaugmentation treatment tests are always critically assessed in terms of efficiency and effectiveness. In most cases, the inocula are usually highly active and efficient in removal of the contaminants under laboratory conditions; whereas, it is difficult to predict their performance under natural conditions, and very little is known about actual cell densities and spatial distribution over the duration of the bioaugmentation treatment test. Hence, the objective of this research was to develop a quantitative SYBR Green I real-time PCR assay based upon 16S rRNA genes to provide assessment of the abundance and distribution of a bioaugmented culture (stain 183BP) in 1,1,1-TCA biodegradation tests conducted at the Moffett field, CA. Additionally, the groundwater bacterial community structure was characterized and analyzed for significant community shifts corresponding to butane and oxygen enrichment and 1,1,1-TCA biodegradation using the terminal restriction fragment length polymorphism (T-RFLP) methods and statistical analysis.

4.2 Materials and methods

4.2.1 Site description

The Moffett Federal Airfield In-Situ Bioremediation Test Site (Moffett Field), Mountain View, CA is located on the lower part of the Stevens Creek alluvial fan, 3 km south of the San Francisco Bay. This site was contaminated with various chlorinated aliphatic hydrocarbons (CAH) and has been used to evaluate in-situ biodegradation processes for the past twenty years. Robert et al (1990) reported that the geology in Moffett Field from surface to bottom consisted of a 0.5m layer of silty sand with pebbles, a 4m layer of silt and clay, a 1.5m aquifer composed of

fine- to coarse-grained sand and gravel, underlain by a clay zone at the bottom. The groundwater velocity ranges from 1 to 1.5 m^3/day. The average porosity is around 0.33 and pumping tests indicated that the aquifer had a transmissivity of 140m^2/day, a storativity of 0.00013, and an r/B value of 0.05 (Semprini et al, 1988).

Initial site characterization found CAH contamination in the groundwater, including trichloroethene (TCE), 1,1,1-TCA, 1,1-DCE, cis-1,2-dichloroethene (c-DCE), trans-1,2-dichloroethene (t-DCE) and vinyl chloride (VC) ranging from 60 to 250 µg/L (Semprini, 1990). Across most of the site, 1,1,1-TCA was the primary CAH contaminant at concentrations of about 100 µg/L with 1,1-DCA and TCE present in trace amounts (Robert et al., 1990).

4.2.2 Strain 183BP and its growth condition

Kim et al (1997) reported that butane-utilizing microorganisms enriched from environmental samples obtained from the Hanford DOE site, WA, could degrade 1,1,1-TCA, 1,1-DCE and their abiotic transformation products. A maximum concentration of 8310 µg/L 1,1,1-TCA was transformed by the culture enriched from the Hanford, WA, samples. From this enrichement, Rungkamol (2001) developed a mixed culture of butane-utilizing microorganisms and tested it in microcosms containing groundwater and aquifer solids obtained from Moffett Field. The microcosms bioaugmented with the culture maintained long-term (13 days) 1,1,1-TCA transformation in the absence of butane utilization and were able to transform mixtures of 1,1,1-TCA, TCE and 1,1-DCE. The inocula was estimated to have Monod parameter values $K_{S,Butane}$ and $K_{S,TCA}$ of 0.11 mg/L and of 0.37 mg/L, respectively. In a study where Rungkamol's culture was bioaugmented into microcosms containing Moffett Field aquifer solids, Lim (2003) found a maximum transformation yield of 0.025 µmol 1,1-DCE/µmol butane and found the dominant organism in the microcosm microbial community in T-RFLP analysis had a TFL of 183 bp when digested with MnlI restriction endonuclease. Strain 183BP was

isolated from Rungkamol's mixed culture by serial plating and growth on MSM agar with butane supplied in the headspace. It was found that a 1000 bp strand of the 16S rDNA sequence of strain 183BP had 100% identity with *Rhodococcus sp*. USAN-012 (Genebank accession number AF420413), a acetonitrile-degrading, gram-positive actinomycete. Bacterial strain 183BP was used for bioaugmentation in aqueous media, microcosm, and filed studies. The culture was grown in mineral media [$K_2HPO_4 \cdot 3H_2O$, 2030.9mg/L; $NaH_2PO_4 \cdot H_2O$, 739.0mg/L; $MgSO_4$, 60.2mg/L; $CaCl_2$, 11,1mg/L; $NaNO_3$, 153.0mg/L; $FeSO_4 \cdot 7H_2O$, 6283.0µg/L; $MnCl_2 \cdot 4H_2O$, 300.8µg/L; $ZnSO_4 \cdot 7H_2O$, 146.6µg/L; H_3BO_3, 61.8µg/L; $Na_2MoO_4 \cdot 2H_2O$, 108.9µg/L; $NiCl_2 \cdot 6H_2O$, 23.8µg/L; $CuCl_2 \cdot 2H_2O$, 17.0µg/L; and, $CoCl_2 \cdot 6H_2O$, 23.8µg/L] with butane supplied in the headspace. To grow the amount of culture required for the field tests (~ 5 g dry wt.), multiple batch vessels each having 300 ml of media and 450 ml of headspace containing approximately 6% butane were used.

4.2.3 Laboratory microcosm study

Mathias (2002) reported that butane-enriched microcosms bioaugmented with strain 183BP showed rapid degradation of 1,1-DCE, followed by slower transformation of 1,1-DCA and 1,1,1-TCA. However, increases in transformation rates of 1,1-DCA and 1,1,1-TCA were observed after most of the butane had been consumed, which indicated strong butane inhibition. No CAH biotransformation was observed in the non-bioaugmented microcosms enriched with butane. However, other microcosm tests conducted with non-bioaugmented Moffett aquifer solids and groundwater showed 1,1-DCE transformation, but no transformation of either 1,1-DCA or 1,1,1-TCA (Rungkamol, 2001).

Indigenous and bioaugmented microcosms constructed with groundwater and aquifer solids from the Moffett Field test site were tested for butane utilization and 1,1-DCE transformation and analyzed for microbial community structure using the T-RFLP method (Lim, 2003). The results showed that strain 183BP was

dominant in bioaugmented microcosms that could utilize butane as growth substrate and rapidly transform 1,1-DCE, a toxic byproduct of abotic 1,1,1-TCA transformation. Non-bioaugmented microcosms transformed significantly less 1,1-DCE and had with no evidence of strain 183BP in the microbial community based on T-RFLP analyses.

4.2.4 Test site layout and operation

Figure 4.1 represents a plan view and a cross-sectional view of the well field used in this study. There were two hydraulically independent well legs that were operated simultaneously at the site. Both well legs received the same chemical amendments, but only the east well leg was bioaugmented. Each leg contained an injection well and an extraction well 7m apart. Groundwater monitoring wells (S1, S2, S3) were located 1.0, 2.2, and 4.0m from the injection wells. Two fully-penetrating wells (S0.5, S1.5) containing stainless steel mesh coupons filled with glass beads (3mm in diameter) or quartz sand (1mm in diameter) were located 0.5m and 1.5m from the injection well.

Groundwater injection and extraction rates were about 1.5 and 2.0 L/min, respectively. The groundwater extraction flow rate was controlled by variable-frequency pumps and measured by an electronic paddle-wheel sensor, while the injection flow rates were controlled by variable speed gear pumps and rotometers. Groundwater from the extraction well was chemically amended and re-circulated back to the injection well. Unused extraction water was air-stripped and disposed to the sewer. The injection system was equipped with solenoids and a pulse timer allowing alternate butane- and oxygen-purging of the recirculated groundwater to eliminate the possibility of developing explosive gas mixtures. A data acquisition and control system (DAC) mounted to an automated sampling and analysis platform (ASAP) semi-continuously measured the concentrations of butane, chlorinated aliphatics, pH, anions, and dissolved oxygen in field samples as described by McCarty et al (1998).

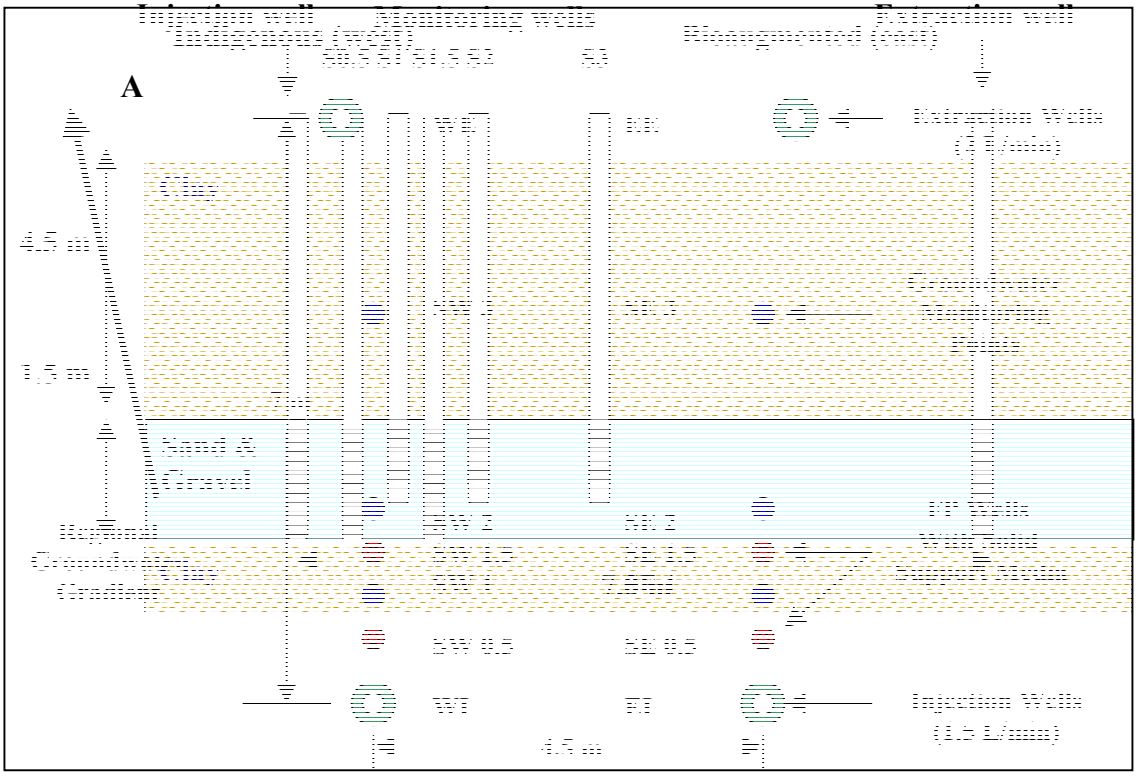


Figure 4.1 Scheme of the test zone. Plan view (A) and vertical section view (B).

4.2.5 DNA extraction from groundwater and solid coupons

Groundwater samples were collected from the monitoring wells and shipped on ice to Oregon State University. Bacterial cells from 250 ml groundwater samples were concentrated on 0.2μm-pore-size white polycarbonate filter membranes (diameter, 25mm; Osmonic Inc., Trevose, PA) with 0.8μm-pore-size nitrocellulose support membrane (diameter, 25mm; Millipore) by applying a vacuum of 20 in. Hg. The polycarbonate filter membrane was then placed into a clean 1.5 ml microcentrifuge tube (Eppendorf, Germany) for subsequent DNA extraction. Occasionally, the presence of small soil and organic particles contained in the groundwater samples caused membrane clogging during filtration. When this occurred, a centrifuge method was used to concentrate the cells for DNA extraction. A 240-ml portion of the water sample was equally dispensed into 6×50 ml centrifuge tubes (VWR International, San Diego, CA) and centrifuged at 5000×*g* for 10 min. The pellet from each tube was transferred to a clean 1.5 ml microcentrifuge tube (Eppendorf, Germany) and centrifuged at 9000×*g* for 3 min. All the resulting pellets were placed into a new 1.5 ml microcentrifuge tube (Eppendorf, Germany). After another centrifuge step at 9000×*g* for 3 min, supernatant was removed and the remaining pellet was taken through the DNA extraction procedure.

All the resulting concentrated cell samples underwent DNA extraction using DNeasy Tissue Kits (Qiagen, Valencia, CA) and the supplied protocol for DNA extraction from gram-positive organisms. After extraction, DNA samples were stored at -20 ºC for future analyses.

4.2.6 Primer design and specificity

Potential 16S rDNA-targeted oligonucleotide primers applicable to the specific detection of strain 183BP were formulated using the probe design tool of the ARB program package (written by O. Strunk and W. Ludwig; available at http://www.arb-home.de). It is a graphically-oriented software package for phylogenetic treeing, sequence data analysis and molecular probe design. The core database used was constructed from a 16S rRNA database downloaded from RDP II (Hugenholtz, 2002; available online at http://rdp.cme.msu.edu/html/alignments.html), which contains more than 8000 sequences, most of them belonging to soil bacteria.

Since the 16S rDNA sequence of strain 183BP shares significant similarity with other bacterial strains, 14 closely related 16S rDNA sequences were used in designing the specificity of the 183BP-specific primers. The probe design parameters were defined as follows: G+C content (50 to 100%), corresponding E. coli. number (0 to 1000), maximal hairpin bonds (0), melting temperature (30 to 100 ˚C), and primer length (18-mer).

Two resulting primers, Ran191F (5'-GTT CCG GGG TGG AAA GGT-3') and Ran443R (5'-ACT CGC GCT TCG TCG GTA-3'), were chosen for use in this study. The specificity of the two newly designed primers for the 183BP strain was confirmed using the PROBE_CHECK tool of the ARB program, the PROBE_MATCH tool of the RDP II, and the BLAST search tool provided by NCBI. It was shown that the 183BP-specific primer pair had complete identity to sequences of six other known bacterial strains (Table 4.1).

Table 4.1 Bacterial strains that can be amplified by the 183BP-specific primer pair (Ran191F and Ran443R).

Genbank Accession #	Microorganisms that can be amplified by the specific primer pair
AF420413	*Rhodococcus sp*. USA-AN012
AF447392	*Rhodococcus aetherovoranes strain* Bc663
AF447391	*Rhodococcus aetherovoranes strain* 10bc312
AF103733	*Rhodococcus sp*. YH1
AF350248	*Rhodococcus rubber strain* AS4.1187
AJ459106	*Rhodococcus sp.* HN

4.2.7 PCR amplification of extracted DNA

The extracted DNA was amplified using the 183BP-specific primers (Ran191F and Ran443R) to confirm the presence of strain 183BP in the samples. Additionally, two universal bacteria primers 27F-B (5'-AGR GTT YGA TYM TGG CTC AG-3') and 338Rpl (5'-GCW GCC WCC CGT AGG WGT-3') were used to prepare the samples for T-RFLP analysis. The 27F-B primer was labeled on the 5' end with a FAM dye for T-RFLP analyses. PCR reaction mixtures contained 20 ul 2.5×PCR Eppendorf MasterMix (Eppendorf, Westbury, NY) to provide final concentrations in the PCR reaction of 1.25U *Taq* DNA polymerase, 50 mM KCl, 30 mM Tris-HCl, 1.5 mM Mg2+, 0.1% lgepal-CA630 and 200 µM each dATP, dCTP, dGTP and dTTP, 20 – 40 pmol of each primer, and 2 µl of template DNA. All PCR amplifications were performed with an Eppendorf Gradient Thermocycler (Eppendorf, Germany) and were carried out in a final volume of 50 µl. The PCR reaction conditions consisted of one cycle at 95 ºC for 2.5 min, 55 ºC for 1 min, and 72 ºC for 2 min; 35 cycles at 95 ºC for 0.5 min, 55 ºC for 1 min, and 72 ºC for 1 min; and a final extension at 72ºC for 20 min and cooling at 4ºC. The annealing temperature (55 ºC) was the same for all the primers.

Five microliters of each resulting PCR product was visualized on a 1% agarose gel (EM Chemical) stained with 0.5 μg/ml ethidium bromide. MassRuler Low Range DNA Ladder (MBI Fermentas Inc, Hanover, MD) was loaded as a DNA mass marker. The DNA Ladder yields the following 11 discrete fragments (in base pairs): 1031, 900, 800, 700, 600, 500, 400, 300, 200, 100, and 80. Electrophoresis of 5μl MassRuler DNA Ladder resulted in bands containing 50, 45, 40, 35, 30, 50, 20, 15, 10, 5, 4 ng of DNA, respectively. The agarose gel was run in 1×TAE (0.04M Tris-acetate, 0.001M EDTA) electrophoresis buffer at 220 volts for 40 min.

The gel electrophoresis of PCR products was imaged using a BioDoc-It system (UVP Inc, Upland, CA). The DNA band intensities in the electronic images were then analyzed using NIH Image J program (written by Wayne Rasband of the National Institute of Health; available at http://rsb.info.nih.gov/ij/ for free) and compared to the DNA mass standards.

4.2.8 Real-time SYBR Green PCR

Real-time PCR was performed using the 183BP-specific primers in a Sequence Detection System 7000 (PE Applied Biosystems, Foster City, CA). The SYBR Green PCR Master Mix (PE Applied Biosystems, Foster City, CA) contains SYBR Green I Dye, AmpliTaq Gold® DNA polymerase, dNTPs (containing dUTP), Passive Reference 1, and optimized buffer components. All amplification reactions were carried out in a total volume of 25 μL in a 96-well optical reaction plate with optical adhesive covers (PE Applied Biosystems, Foster City, CA). Each reaction contained 1 μL of template DNA, 12.5 μL of SYBR Green PCR Master Mix (2X conc.), 150 nM concentrations of forward and reverse primers, and, to reach a total volume of 25 μL, molecular biology grade water. The cycling parameters were initial incubation at 50 ºC for 2 minutes to activate the primers and 95 ºC for 10 minutes to activate the *Taq* polymerase, followed by 40 cycles of 95 ºC for 15 seconds and 60 º C for 1 minute. A primer titration was performed using

50/50, 150/150, 300/300 nM concentrations of 183BP-specific primers to determine the optimum primer concentrations.

For this study, an absolute quantitation method was used. Calibration curves were run in parallel and in triplicate with each analysis using the DNA extracted from a pure strain 183BP culture containing 4.0×10^8 cells/mL based on DAPI-stained total cell counts. The extracted DNA was serially diluted down to 10^{-6} corresponding to cell concentrations of 4.0×10^8 cells/mL down to 4.0×10^2 cells/mL. Wells with no DNA template were used as negative controls. The cycle threshold (C_T), indicative of the quantity of target gene at which fluorescence exceeds a preset threshold, was set above the baseline where there was little change in the fluorescence signal. After exceeding the threshold, the sample was considered positive.

Amplification data collected by the 7000 Sequence Detector was then analyzed using the Sequence Detection System software (version 1.1; PE Applied Biosystems, Foster City, CA). The calibration curve was created by plotting the cycle threshold (C_T) against the log of the cell concentrations of each standard dilution. The coefficient of linear regression (R) for each standard curve was calculated.

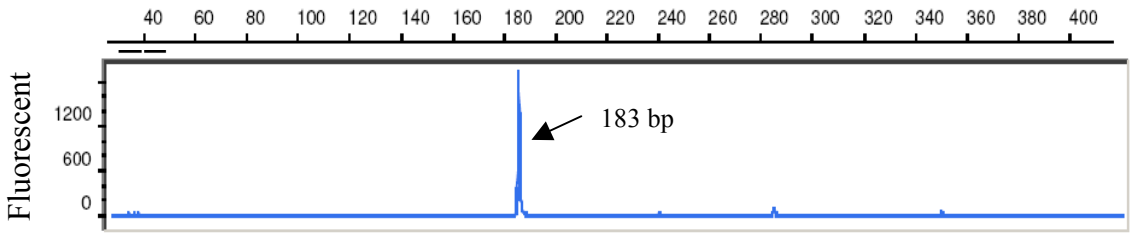
Following amplification, a melting curve analysis was performed to verify the product specificity by its melting temperature (T_m) (Ririe et al, 1997). If SYBR-Green-I-bound dsDNA is heated up, a rapid loss of SYBR Green I fluorescence can be observed near the denaturation temperature. The melting curve and the value of T_m are dependent on the GC content, length and sequence of the amplicon. Thus, specific amplicons can be distinguished by the melting curve analysis. In the present study, T_m was determined during a phase of slow heating from 60 to 95ºC, during which time there occurs a rapid decrease in the fluorescence due to the denaturation of the amplicons resulting in the formation of single filaments of DNA and the successive detachment of the SYBR Green I dye. The melting curves were visualized with Sequence Detection System software

(version 1.1; PE Applied Biosystems, Foster City, CA). Curves with more than one evident melting point were not used for sample analyses.

The optimum primer concentration chosen for the SYBR real-time PCR assay was 150 nM based on trial runs using a range of primer concentrations. This concentration was selected because it provided low C_T values and minimized primer-dimmer formation. Initially, real-time PCR was performed on triplicates of each sample at three serial dilutions in the same plate, 1:1, 1:10 and 1:100. When PCR inhibition did not occur, the C_T values of 1:1 and 1:10 dilutions were very similar. However, the presence of humic or other background compounds in the environmental samples often inhibited the PCR reaction in the 1:1 dilutions. A 10-fold decrease of 183BP cell concentrations was observed between 1:10 and 1:100 dilutions for most samples. Due to the high variability associated with 1:100 diluted samples and the inhibition present in many 1:1 dilutions, samples of 1:10 dilution were chosen for cell quantification.

The purity of the culture used for the real-time PCR standard was confirmed by a DAPI-stained cell morphology check and T-RFLP analysis (Figure 4.2). A standard curve was generated for each 96-well plate analyzed by plotting the C_T values against the corresponding cell densities of 183BP pure culture corresponding to each extracted DNA dilution (Figure 4.3). There was a higher degree of error associated with low cell density samples. For example, the standard deviation for the C_T values was 0.2 cycles for cell densities ranging from 4.0×10^4 cells/ml to 4.0×10^5 cells/ml, whereas the standard deviation for C_T values ranged from 1.9 to 2.5 cycles for densities ranging from 4.0×10^2 cells/ml to 4.0×10^3 cells/ml. Although weak signals could be detected from diluted DNA standards corresponding to cell densities of 40 cells/ml, the high variability associated with C_T from samples containing low cell numbers precluded our ability to distinguish the cell populations in these samples. Only when the template DNA in the PCR mixture came from samples containing 4.04×10^2 cells/ml or greater were we able to reliably differentiate cell concentrations. The C_T values increased with each 10-fold

dilution of the target DNA. Linearity between the C_T values and target concentration was observed over the entire 6 orders-of-magnitude dilution series. An increase in variability with cycle number was observed. Based on triplicate samples, standard deviations were calculated for each sample. Among the negative control samples, some showed a very low fluorescence, but below the threshold cycle level.



Figure

Figure 4.2 T-RFLP profile generated with DNA extracted from the pure culture used for 183BP DNA standard

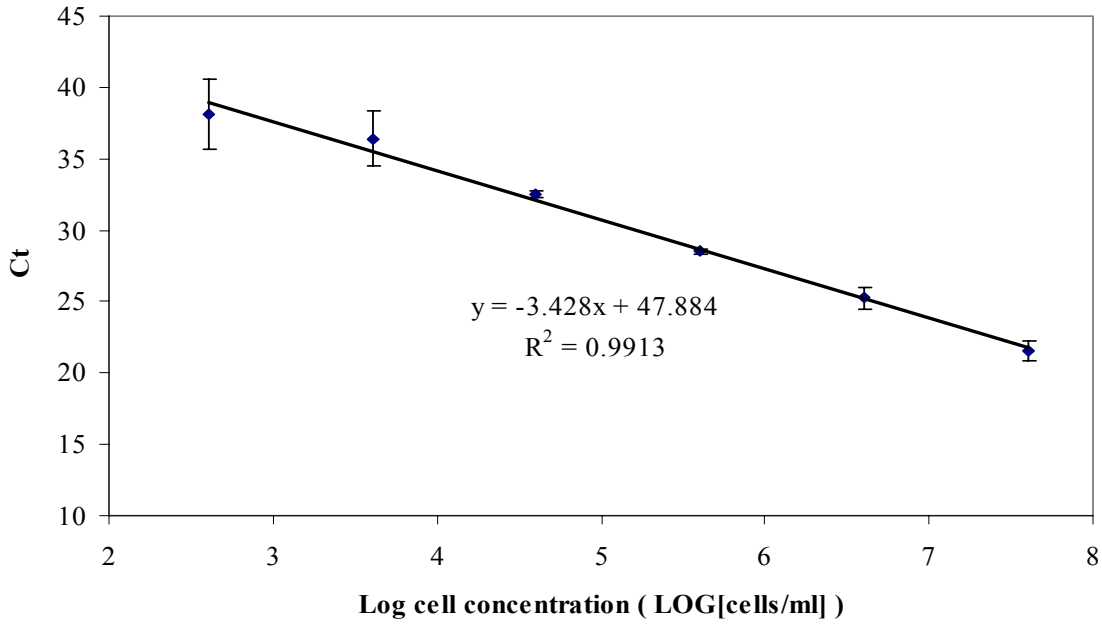


Figure 4.3 183BP DNA calibration curve

4.2.9 Real-time PCR melting curve analysis

The specificity of PCR products amplified from the environmental samples was validated by melting curve analysis. Primers Ran191F and Ran 443R were designed to amplify a 264-bp amplicon, which has a distinct melting temperature of 88.3 ºC (Figure 4.4). The primer titration test showed the melting temperature of a primer dimmer was about 75 ºC. Out of all of the groundwater samples analyzed in this study, only one, collected from the SE0.5 well on day 11, produced significant multiple peaks during melting curve analysis indicating production of non-target amplicons. A duplicate PCR test performed confirmed the presence of these unknown amplification products in the sample, so the sample was not included in data analyses.

4.2.10 T-RFLP analysis

PCR product amplified using the universal bacterial primers was digested at 37 ºC for 4.5 hours with *Mnl*I or *Hin6*I restriction endonucleases (MBI Fermentas Inc, Hanover, MD) followed by incubation at 80ºC for 10 min to terminate enzyme activities. The recognition sites of these enzymes and the composition of the reaction mixture are shown in Table 4.2.

Products of the restriction digestion were diluted to an appropriate level and analyzed on an ABI 377 slab gel platform with Filter set A, as the dye set consisted of 6-FAM (blue) labeled samples and a Rox (red) standard. GENESCAN400-ROX (fragment size: 50-, 60-, 90-, 100-, 120-, 150-, 160-, 180-, 200-, 220-, 240-, 260-, 280-, 290-, 300-, 320-, 340-, 360-, 380- and 400-bp; PE Applied Biosystems, Foster City, CA) was added into each sample as an internal lane standard. Fragment size was

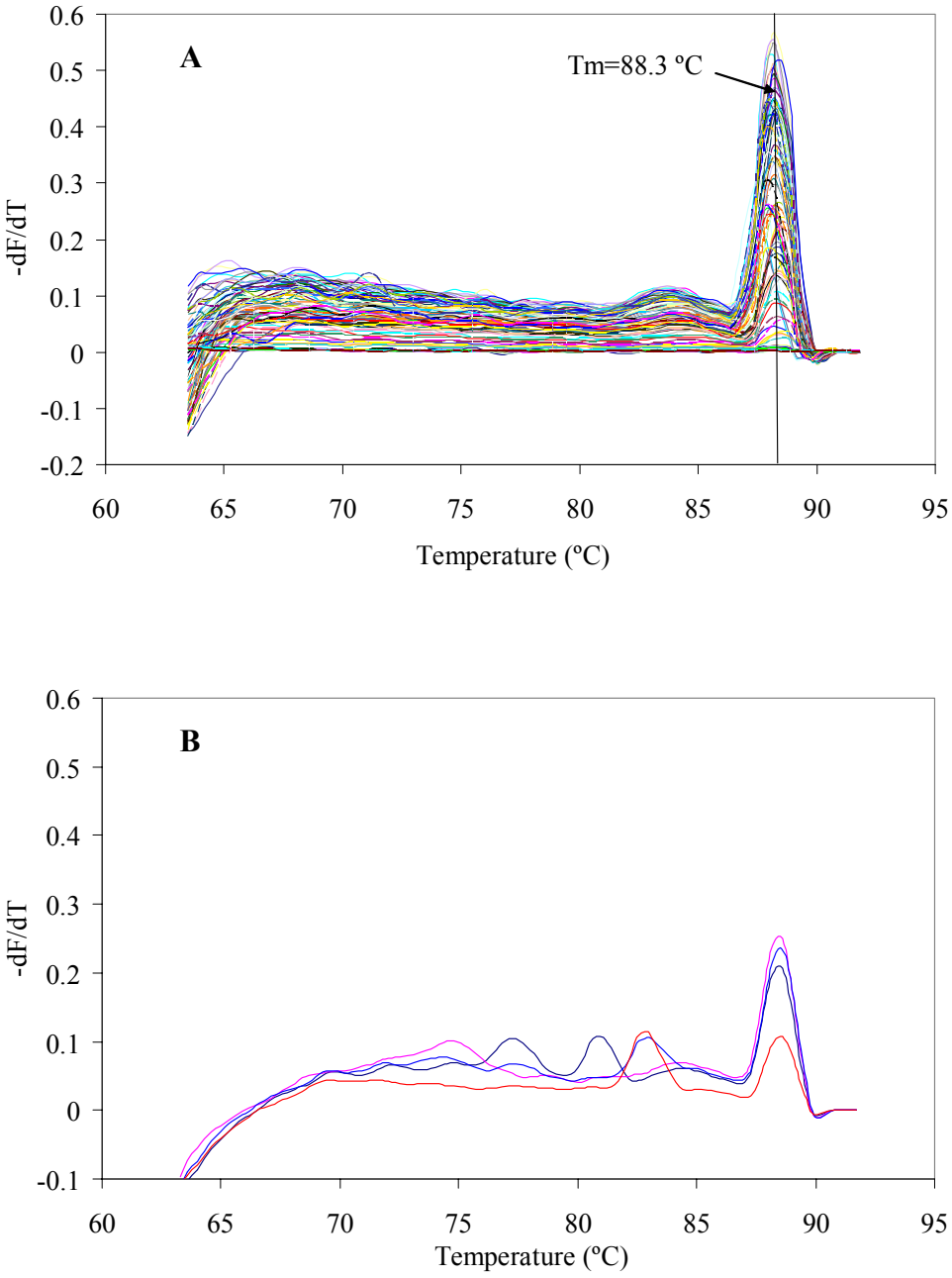


Figure 4.4 Real-time PCR melting curve analysis. Panel A (pure 183BP cells and groundwater samples of no non-target amplicon in PCR reactions). Panel B (groundwater samples of non-target amplicons in PCR reactions)

determined using GeneScan software, version 2.1 (PE Applied Biosystems, Foster City, CA). Results were obtained in the form of an electropherogram and a data table of fragment size, peak height, and peak area.

Table 4.2 Enzyme recognition sites and reaction components

MnlI		Hin6I	
Recognition sites			
5'-C C T C(N)$_7$ ^-3' 3'-G G A G(N)$_6$ ^-5'		5'-G^C G C-3' 3'-C G C^G-5'	
Reaction mixture ingredients			
Reagent	Volume (µl)	Reagent	Volume (µl)
Enzyme (10 U/µl)	0.25	Enzyme (10 U/µl)	0.25
G^+ Buffer	2	Y^+ / Tango Buffer	2
PCR product	10	PCR product	10
Water	7.75	Water	7.75

The densitometric curve of each T-RFLP file was transferred to GelComparII program (version 3.5, Applied Maths Inc., Belgium). Each gel was normalized according to the internal size standard and the banding patterns were compared using the unweighted pair group method with arithmetic means (UPGMA) clustering and the Dice coefficient method. UPGMA is a clustering method based upon the average value of similarity between the two clusters being merged (Romesburg, 1984). Dice coefficient measures the association between any given species without using any species as a base (Dice, 1945).

4.2.11 Total cell counts

Total cell counts were conducted by filtering 10 ml samples through 0.2 µm pore size white polycarbonate filter membranes (diameter, 25 mm; Osmonic Inc.) with 0.8 µm pore size nitrocellulose membrane support (diameter, 25 mm; Millipore Inc.) and glass microfibre filter backing (diameter, 25 mm; Whatman) by applying a slight vacuum of ~5 in. Hg. After filtration, the polycarbonate

membrane was transferred from the filter stack to a microscope slide (size, 25×75×1 mm; Fisher Scientific) using Cryo-Babies (Diversified Biotech), and air-dried in the dark. The slide was transferred into DAPI-staining buffer (5 μg/ml DAPI, 150 mM NaCl, 20 mM Tris/HCl [pH 7.4], 5 mM EDTA, and 0.01% sodium dodecyl sulfate), incubated for 10 minutes in the dark, rinsed with DAPI-washing buffer (150 mM NaCl, 20 mM Tris/HCl [pH 7.4], 5 mM EDTA, and 0.01% sodium dodecyl sulfate) for another 2 minutes, and finally air dried in the dark with the Cryo-Babies stickers removed. A drop of Citifluor antifadent (Ted Pella, Inc., CA) was added to each membrane which were then covered with a cover slip, sealed with nail polish, and stored at –20 °C with desiccant.

DAPI-positive cells were counted for each field of view using a DMRB epifluorescence microscope (Leica Inc, Germany) coupled with an ORCA-ER CCD digital camera (Hamamatsu Inc., Japan) with a filter set appropriate for DAPI. Consistent exposure time for DAPI was 5 seconds. DAPI images were then processed by IPLab software (version 3.5.5, Scanalytics Inc, Fairfax, VA).

Total cell counts and FISH-labeled cell counts were calculated by the method developed from Kepner and Pratt (1994). Cell counting was performed on randomly chosen fields of view (FOV) for each test sample. The number of target cells per milliliter of groundwater sample was determined from values for the effective area of the filter, the area of the FOV, the dilution of the sample applied, and the volume of sample filtered, as follows:

$$\text{Total number of target (cell/ml)} = (N \times A_t)/(d \times V_f \times A_g)$$

Where, N: mean number of cell counted per FOV;

A_t: effective area of the filter (mm^2)

d : dilution factor (V_{final}/V_{sample})

V_f : volume of diluted sample filtered (ml)

A_g: area of FOV (mm^2)

4.3 Results

4.3.1 Degradation of chlorinated ethanes

The bioaugmentation test began on October 5, 2003. The concentrations of bromide, 1,1,1-TCA, 1,1-DCA, butane, and dissolved oxygen (DO) in the two injection wells (EI and WI) and the monitoring wells (SE1, SE2, SE3, SW1, SW2 and SW3) were monitored continuously for the duration of the test. Each sample required approximately 50 min to acquire, prepare, and analyze, resulting in approximately 25 samples and 3 standards run each day. One hour prior to bioaugmentation, oxygen-purged groundwater was introduced to both well legs. Approximately 5 g dry wt. of a mixed butane-utilizing culture was diluted in 4 L of groundwater and introduced into the injection stream to the east well leg over a period of ten minutes. Immediately after bioaugmentation, chemical amendment of both well legs ensued. 1,1,1-TCA and 1,1-DCA were introduced continuously at approximately 200 μg/L and bromide was added at approximately 130 mg/L. Alternate pulses of butane-purged and oxygen-purged groundwater at influent concentrations of about 22 mg/L and 40 mg/L, respectively, were also initiated. Pulse cycles were 9 minutes of butane purging followed by 4 minutes of nitrogen purging, 156 minutes of oxygen purging, and another 4 minutes of nitrogen purging before repeating the cycle. In this way explosive gas mixtures were not formed and the alternating feed encouraged microbial growth farther from the injection well.

Bromide breakthrough in the bioaugmented east leg followed the pattern of 100% capture in SE1 and approximately 86% to 89% recovery in SE2 and SE3, respectively (Figure 4.5). In the indigenous west leg, recovery efficiencies were approximately 87% in wells SW1 and SW3, while well SW2 appeared to be significantly farther out of the flow path with only 19% bromide recovery. Consequently, data from SW2 were not included when analyzing treatment efficiencies in the west leg. It took about 0.4 day, 1.4 day, and 1.6 day to achieve

50% bromide breakthrough at wells SE1, SE2, and SE3 respectively. In the west well leg, 50% breakthrough occurred at about day 0.15 in SW1 and 0.65 d in SW3.

Organisms in the non-bioaugmented west leg took 9 days to grow to sufficient density to completely utilize the added butane by the first monitoring well (Figure 4.6). In contrast, only 3 days were required to achieve the same result in the bioaugmented east leg. An approximate time-averaged butane concentration of about 3 mg/L was reached in the west leg prior to complete butane utilization. In the bioaugmented leg, immediate butane utilization resulted in a transitory maximum concentration of about 2.5 mg/L at the first monitoring well with significantly lower concentrations in wells SE2 and SE3. After initial enrichment, butane concentrations remained very low throughout the field for the duration of the test, with one exception. Beginning on day 24, butane delivery to the field was interrupted for 2 days. Upon restart of butane addition, butane levels in the field rose to approximately 500 μg/L in SE1 and about 3 mg/L in SW1 before the butane-utilizing microbial population responded and reduced concentrations to near zero values again. Similar to the initial start-up period, this process was much quicker in the bioaugmented leg than in the indigenous leg.

Oxygen utilization in both well legs occurred primarily between the injection well and the first monitoring well (Figure 4.7). Oxygen utilization was fairly stable throughout the test, except for transient declines in concentration around day 3 when oxygen delivery to the field was interrupted for about one day. Oxygen utilization averaged about 20 mg/L in the bioaugmented east leg and about 24 mg/L in the west leg throughout the first 20 days of the test, dropping to about 12 - 13 mg/L in both legs from day 20 to day 30 of the test. The apparent slight utilization of oxygen in the east leg between the first and second monitoring well is an artifact of incomplete capture of the flow path as evidenced by the bromide breakthrough data for the two legs.

In the west leg, TCA and DCA concentrations rapidly approached influent values and remained there throughout the test (Figures 4.8 and Figure 4.9).

Times to 50% TCA and DCA breakthrough were about 0.25 day and 0.9 day for wells SW1 and SW3, respectively. In the bioaugmented east leg, TCA concentrations rose for the first three days followed by a decrease in concentration around day four corresponding to the time to complete butane utilization. During this period of maximum treatment efficiency, approximately 80% of the influent TCA was effectively treated. However, treatment efficiency began a steady decline until about day 21 when essentially all TCA treatment ceased in the east leg. A similar pattern developed for DCA in the east leg with maximum treatment occurring around day 4 to day 7 with about 96% DCA removal efficiency. Treatment efficiencies also slowly declined over time, but not as dramatically as those for TCA. At day 21, about 53% DCA removal efficiency remained; however, by day 26 DCA transformation essentially ceased.

4.3.4 Detection and quantitation of strain 183BP during bioaugmentation

T-RFLP analysis of the mixed bioaugmentation culture showed that strain 183BP accounted for only 13.5% of the total peak area in the T-RFLP profile (Figure 4.12). Although the culture was not dominated by strain 183BP, based on representation in the T-RFLP profile up to 0.68 g dry wt. of strain 183BP was augmented to the east well leg. Microbial sampling was conducted at groundwater monitoring wells SE0.5, SE1 and SW0.5 (Figure 4.10). Prior to bioaugmentation, background groundwater samples taken from the east and west zones revealed that strain 183BP was below the real-time PCR detection limit (~10 cells/mL) in the groundwater. In well SE0.5 groundwater, a significant increase in the 183BP cell density was observed at the first sampling interval 2 days after bioaugmentation. By day 2, the 183BP cell concentration reached its maximum measured concentration of $(1.46 \pm 0.29) \times 10^5$ cells/ml. The concentration declines until day 5 where it begins to level out at about 10^3 cells/ml. Maximum TCA and DCA removal, 80 and 96% respectively, occur during this period from day 4 to day 7.

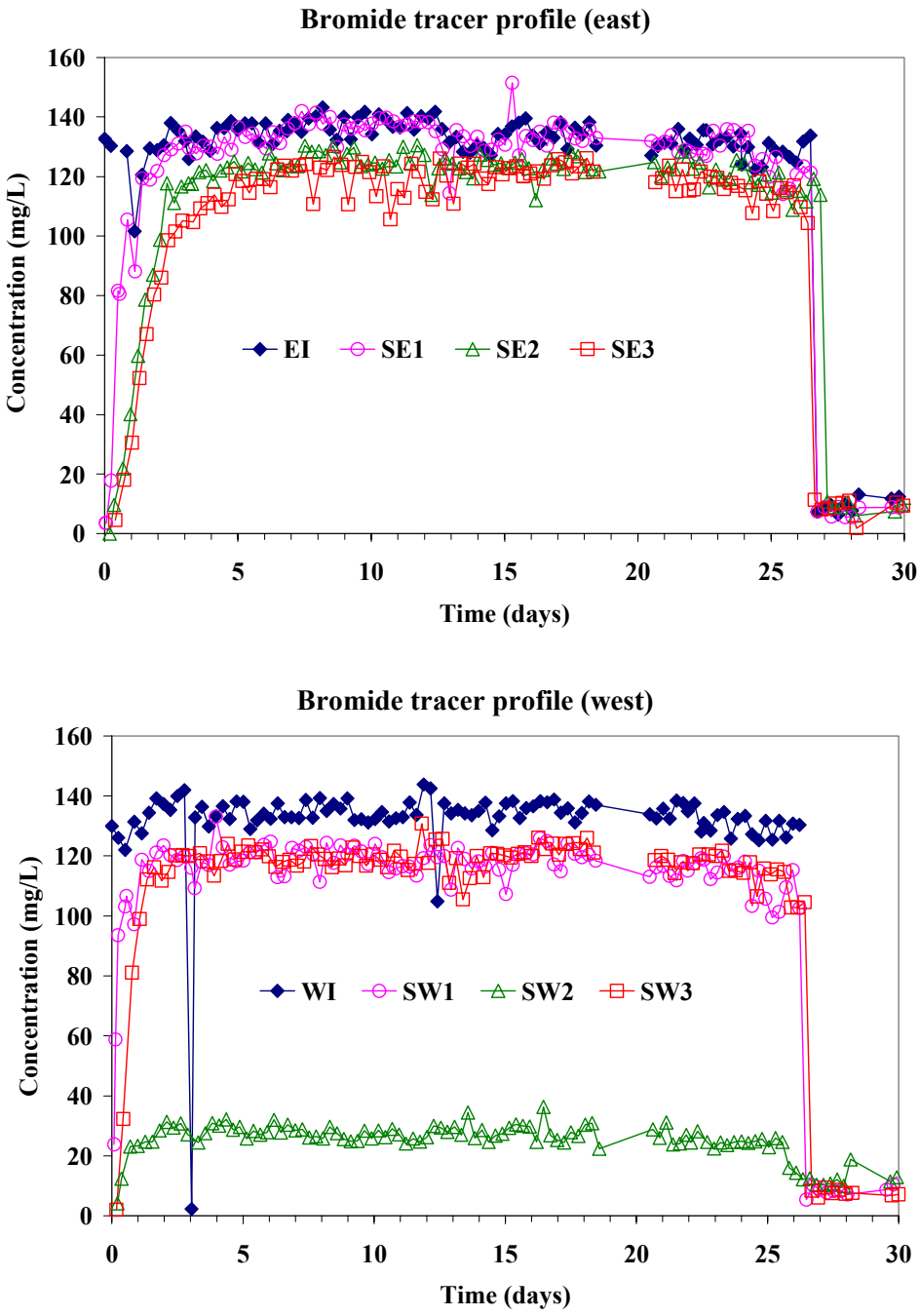


Figure 4.5 Bromide tracer concentrations in the injection wells and the monitoring wells in the two test zones (east, bioaugmented; west, non-bioaugmented) at Moffett Field (data from Gary Hopkins at Stanford University, CA)

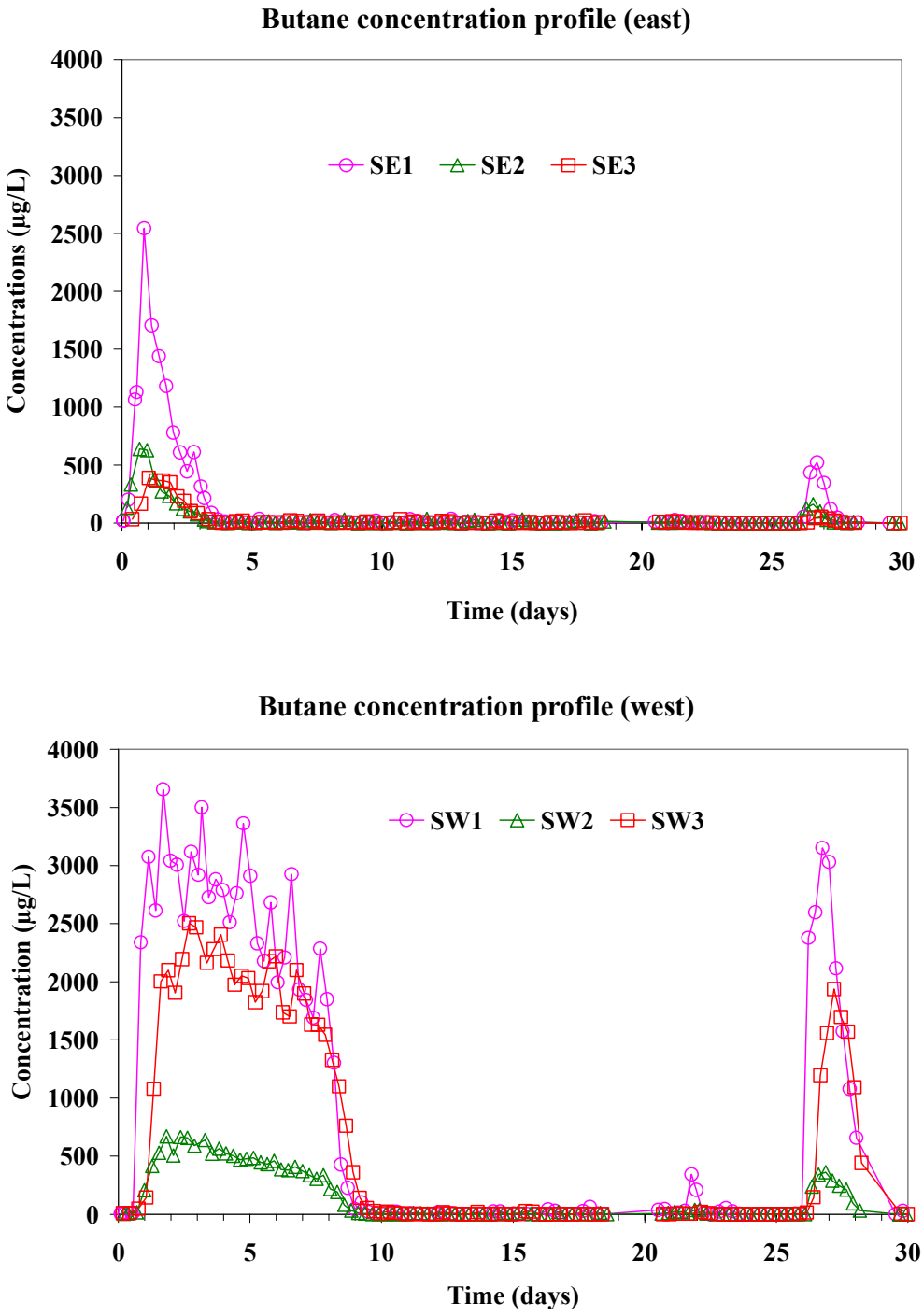


Figure 4.6 Butane concentrations in the injection wells and the monitoring wells in the two test zones (east, bioaugmented; west, non-bioaugmented) at Moffett Field (data from Gary Hopkins at Stanford University, CA)

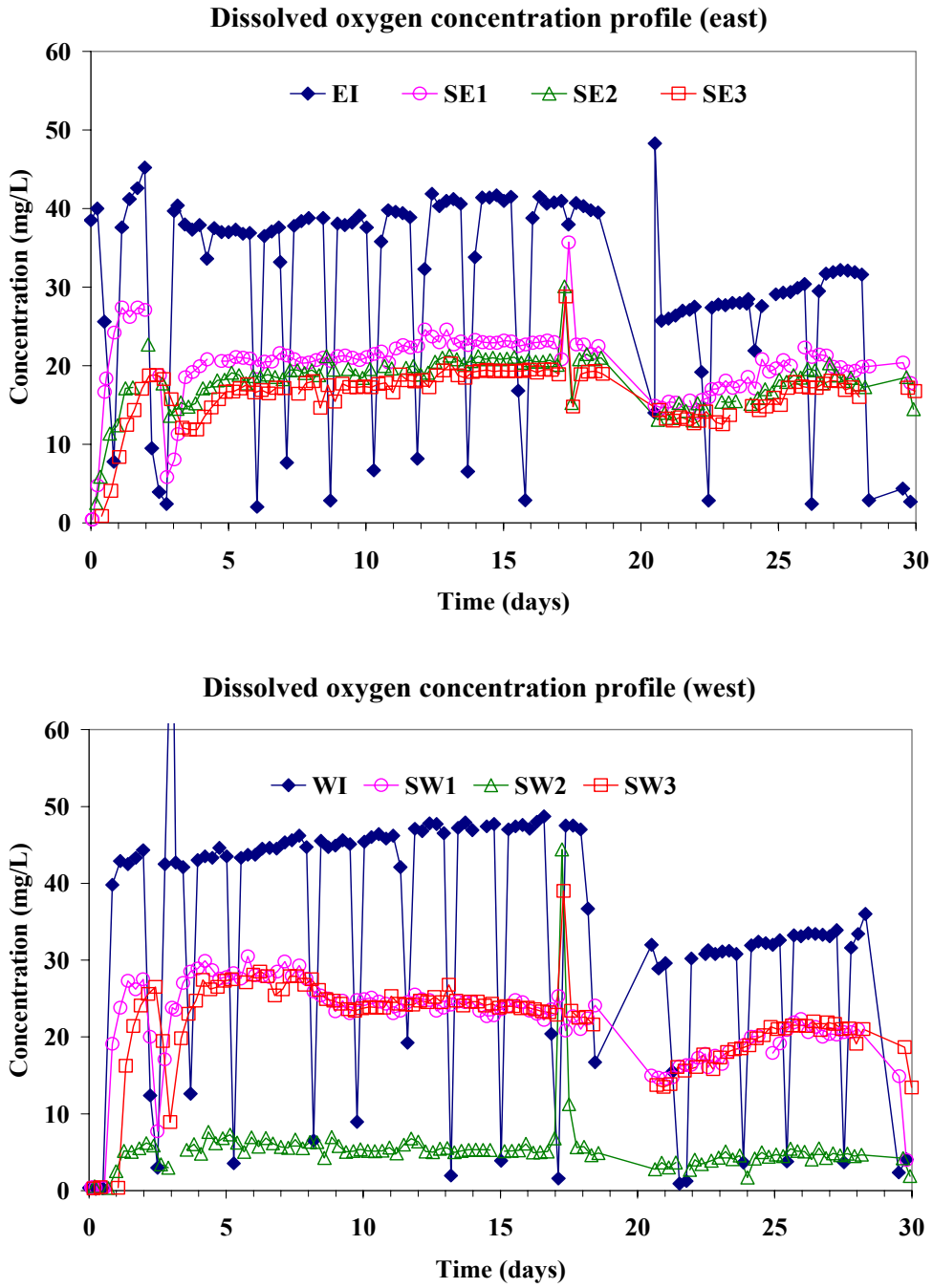


Figure 4.7 Concentrations of dissolved oxygen in the injection wells and the monitoring wells in the two test zones (east, bioaugmented; west, non-bioaugmented) at Moffett Field (data from Gary Hopkins at Stanford University, CA)

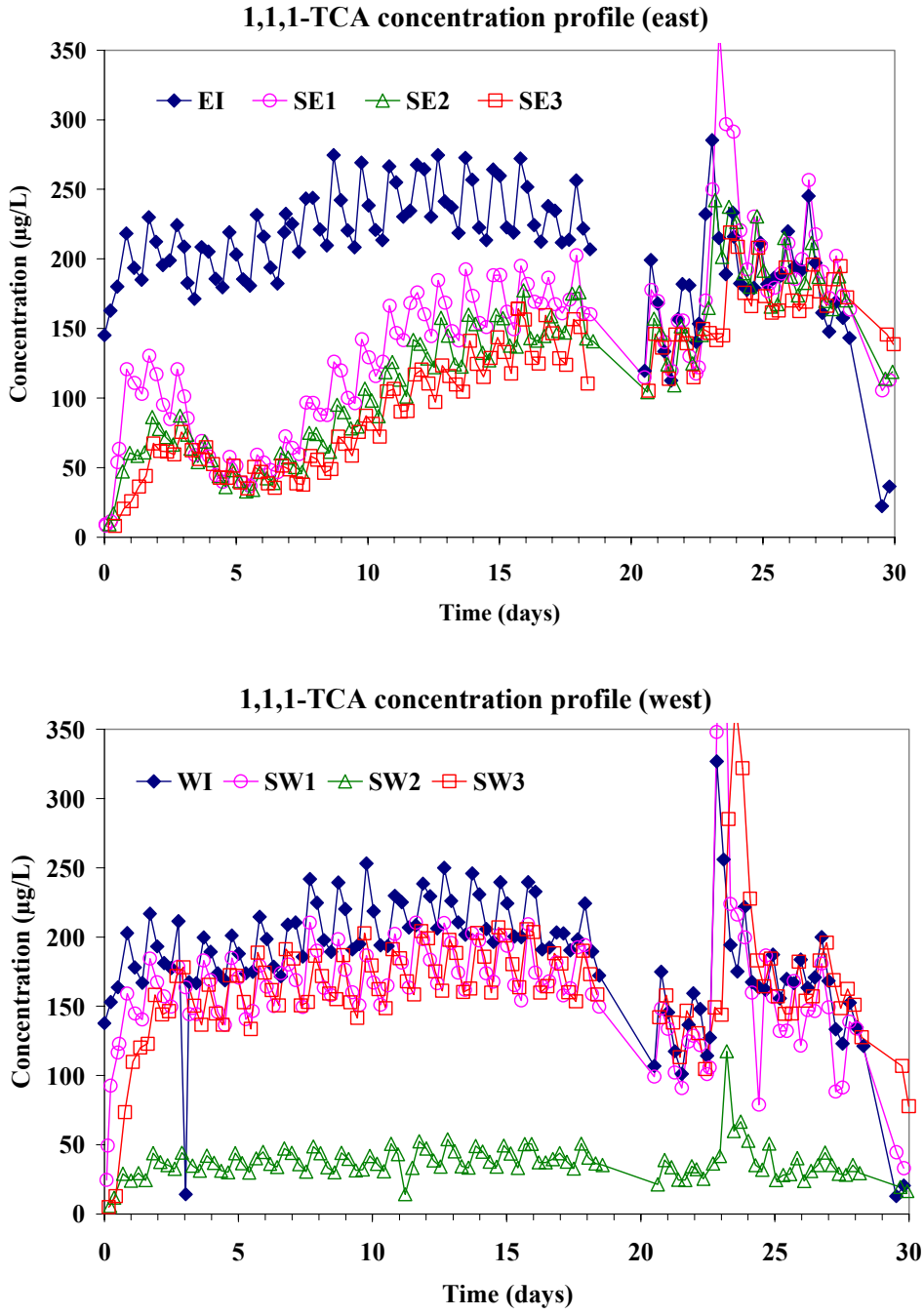


Figure 4.8 1,1,1-TCA concentrations in the injection wells and the monitoring wells in the two test zones (east, bioaugmented; west, non-bioaugmented) at Moffett Field (data from Gary Hopkins at Stanford University, CA)

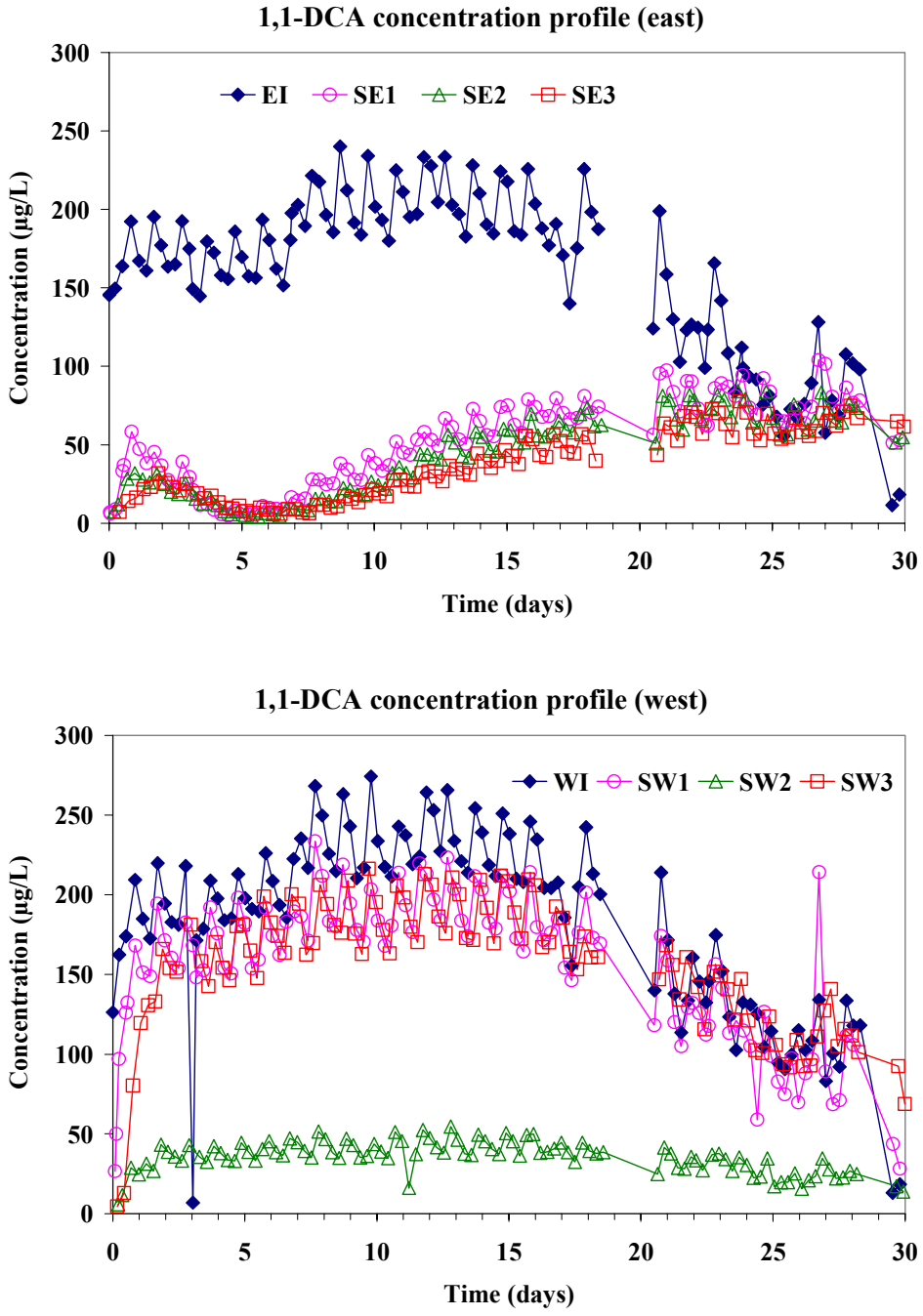


Figure 4.9 1,1-DCA concentrations in the injection wells and the monitoring wells in the two test zones (east, bioaugmented; west, non-bioaugmented) at Moffett Field (data from Gary Hopkins at Stanford University, CA)

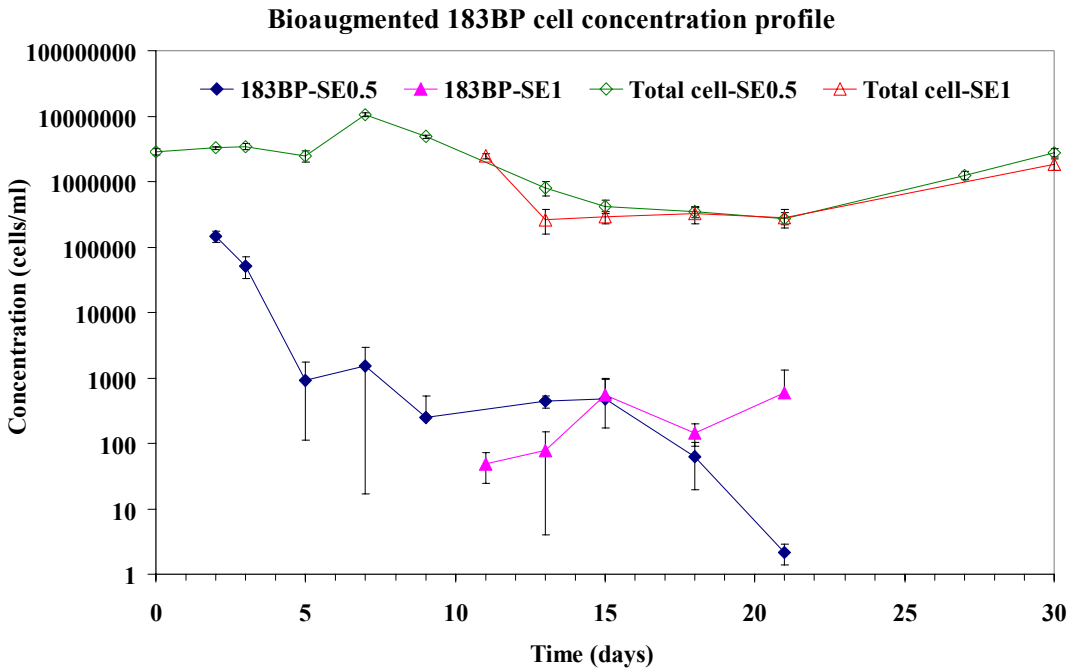


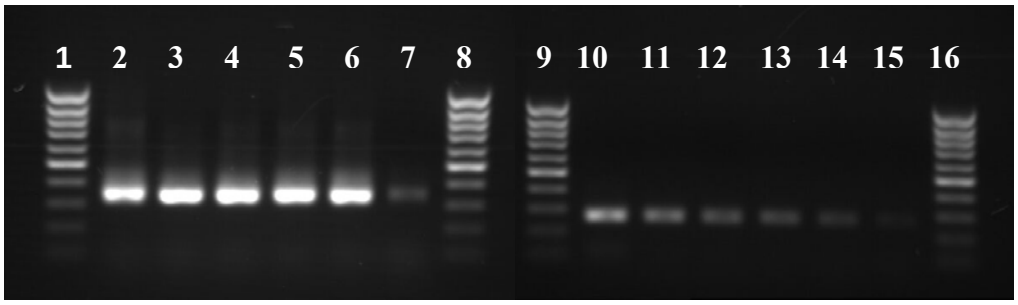
Figure 4.10 Total cell density and strain 183BP cell density profile based on real-time PCR

The population appears to remain relatively stable at cell densities between 250 and 1000 cells/ml through day 15 when another period of cell decline occurs resulting in strain 183BP cell densities below the quantifiable detection limit by day 21. In contrast, 183BP cell densities at the SE1 monitoring well increased from less than 50 cells/ml on day 11 (the first sample taken from SE1) to approximately 600 cells/ml on days 15 and 21; however, the large variance in low cell density measurements precludes placing too much emphasis here. After day 21, no further groundwater samples tested positive for strain 183BP cells. Samples taken from the west well leg before bioaugmentation and on day 21 showed no evidence of 183BP cells.

Figure 4.11 illustrates traditional-PCR-amplified 16S rDNA extracted from the groundwater samples taken from the SE0.5 well from day 5 to 11, and

from the SE1 well on day 11. Quantification of band intensity using NIH ImageJ indicated that the concentrations of 16S rDNA amplified by the universal bacterial primers were approximately 15 ng/µl for all the samples; whereas, a decline in concentration of the 16S rDNA amplified from the 183BP-specific primers was observed in well SE0.5, from 8.5 ng/µl on day 5 to 3.9 ng/µl on day 11. These results are in general agreement with the real time PCR results marking a gradual decline in strain 183BP populations over the extent of the study.

Total cell counts on the same groundwater samples used for real time PCR indicated that the total microbial population in wells SE0.5 and SE1 remained greater than 10^5 cells/ml throughout the test. In well SE0.5, the total cell concentration ranged from a low of $(2.76 \pm 0.56) \times 10^5$ cells/ml on day 21 to a high of $(1.06 \pm 0.846) \times 10^7$ cells/ml on day 7. In well SE1, the total cell concentration showed a similar profile as that of well SE0.5. A clear three-orders-of-magnitude difference between the total cell concentrations and the 183BP cell concentrations was observed throughout most of the test. Only on days 2 and 3 did the 183BP count exceed 1% of the estimated total microbial population with values of 4.5% and 1.5% respectively.



Lane	Sampling date	Location	Primer set
1	80- to 1032-bp DNA mass ruler ladder		-
2	Day 5	SE0.5	27F-B-FAM + Rpl338
3	Day 7	SE0.5	27F-B-FAM + Rpl338
4	Day 9	SE0.5	27F-B-FAM + Rpl338
5	Day 11	SE0.5	27F-B-FAM + Rpl338
6	Day 11	SE1	27F-B-FAM + Rpl338
7	Negative control		27F-B-FAM + Rpl338
8	80- to 1032-bp DNA mass ruler ladder		-
9	80- to 1032-bp DNA mass ruler ladder		-
10	Day 5	SE0.5	Ran191F+Ran443R
11	Day 7	SE0.5	Ran191F+Ran443R
12	Day 9	SE0.5	Ran191F+Ran443R
13	Day 11	SE0.5	Ran191F+Ran443R
14	Day 11	SE1	Ran191F+Ran443R
15	Negative control		Ran191F+Ran443R
16	80- to 1032-bp DNA mass ruler ladder		-

Figure 4.11 Detection of strain 183BP populations in the Moffett groundwater with universal bacterial primers and 183BP-specific primers.

4.3.5 Community T-RFLP profiles

T-RFLP analyses of the bioaugmentation culture and groundwater samples acquired from the field were conducted using universal bacterial primers (27F-B-FAM and 338Rpl) and *Mnl*I and *Hin*6I restriction enzymes. The *Mnl*I T-RFLP profiles provided the highest discrimination of fragments due to increased frequency of recognition sites and the short length of the amplicons used. No fragments of 183 bp length were observed in any field groundwater samples and the other major peaks in the bioaugmentation culture profile at 167-, 174-, 279- and 345-bp accounted for only trace percentages of total peak areas throughout the test period (Figure 4.12).

Before bioaugmentation, T-RFLP profiles of groundwater samples taken from well SE0.5 had major peaks at 120- and 277-bp. In the corresponding non-bioaugmented well leg, SW0.5, at the same time point, the dominant T-RFLP peaks were at 210-, 277-, 279-, and 348-bp. In the bioaugmented well leg, the 277-bp peak accounted for at least 30% of the total peak area on day 0 and day 2 (Figure 4.13). However, the amount of 277-bp fragments declined to less than 1% of the total peak area by day 7. A clear transition occurs from dominance of the 277-bp fragment to dominance of a fragment with a length of 126-bp during the first 20 days of the study.

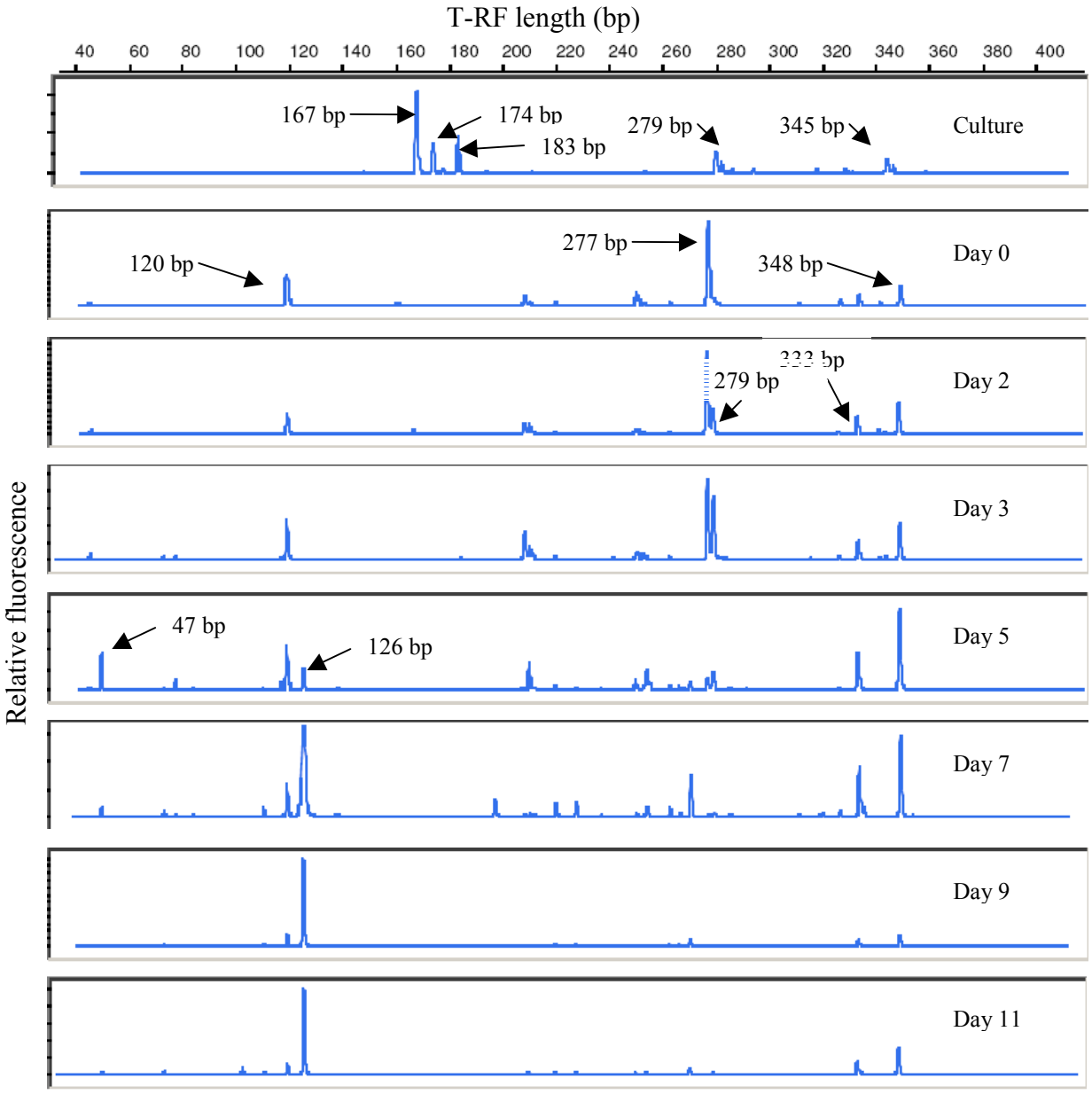


Figure 4.12 T-RFLP profiles generated from the bioaugmentation culture grown in the lab and groundwater samples from well SE0.5 and SW0.5 during the course of bioaugmentation. Universal bacterial primers (27F-B-FAM and 338Rpl) were used in the PCR reactions and the restrictions were performed with the endonuclease *Mnl*I (Fermentas, Inc).

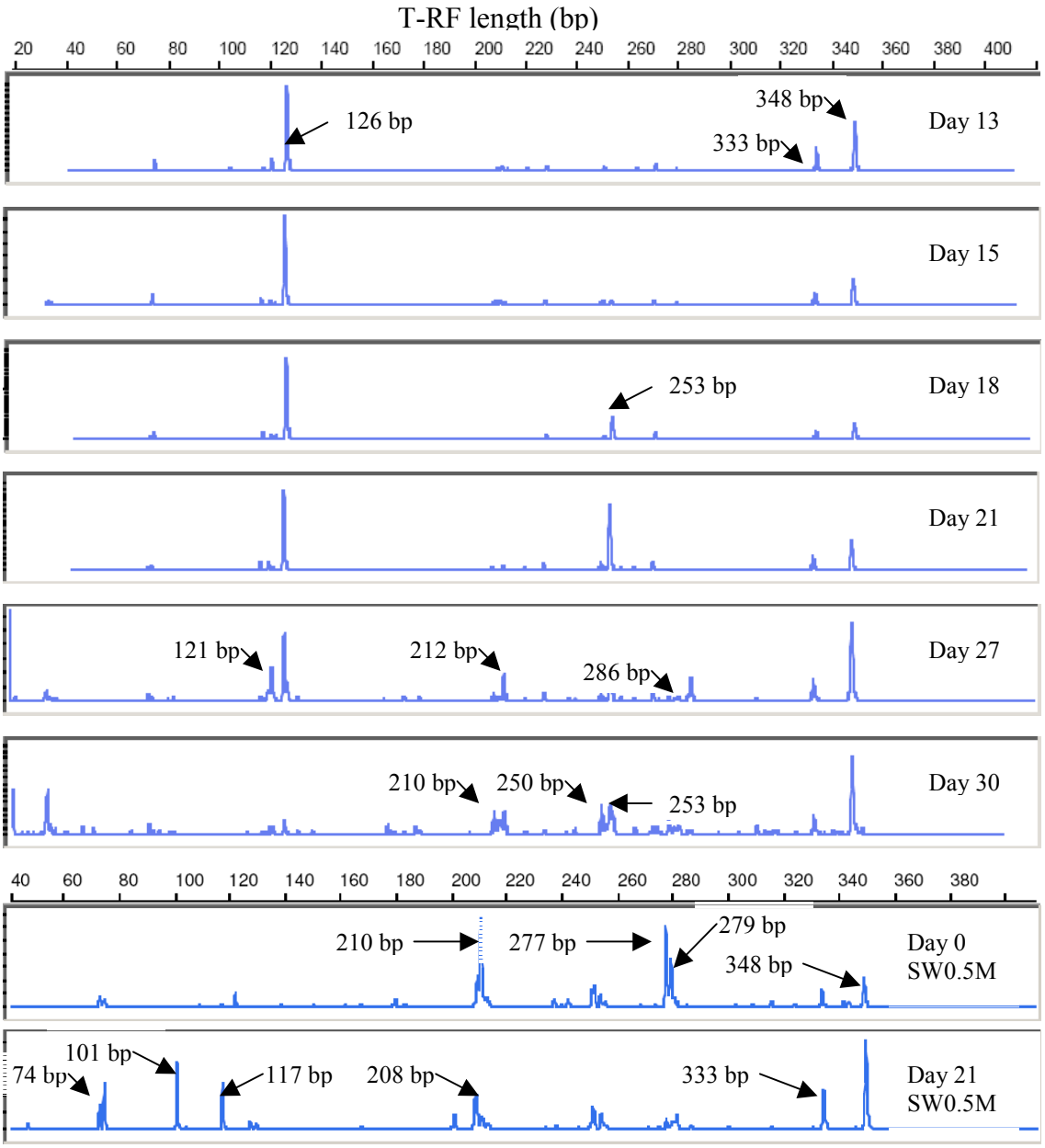


Figure 4.12 (continued). T-RFLP profiles generated from the bioaugmentation culture grown in the lab and groundwater samples from well SE0.5 and SW0.5 during the course of bioaugmentation. Universal bacterial primers (27F-B-FAM and 338Rpl) were used in the PCR reactions and the restrictions were performed with the endonuclease *Mnl*I (Fermentas, Inc).

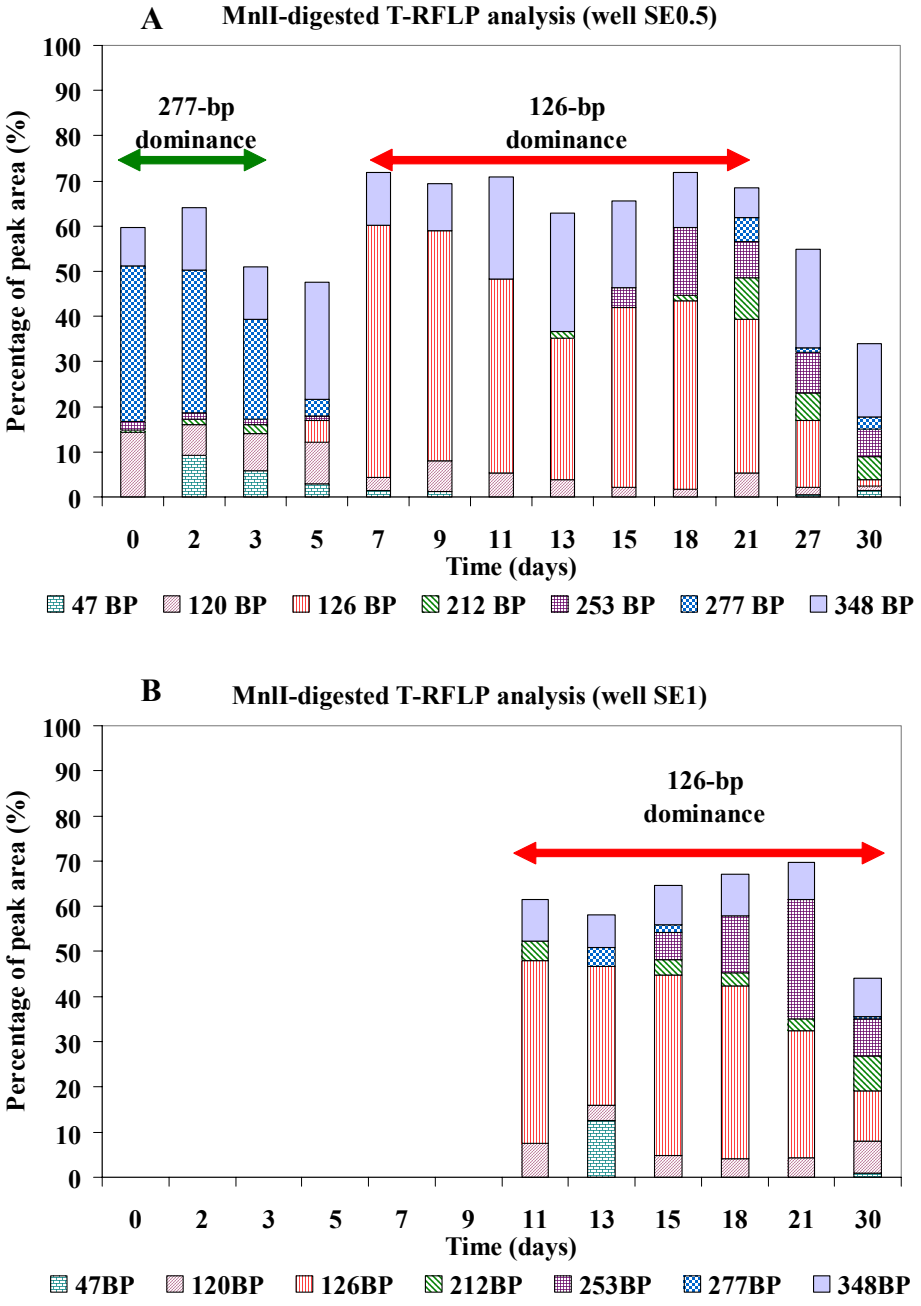


Figure 4.13 Changes in the microbial community structure during the bioaugmentation test in well SE0.5 (A) and SE1 (B).

From day 7 to day 21, the 126-bp peak accounted for at least 30% of the total peak area in all of the samples.

By day 27, a more diverse microbial community appears to have developedas the dominant 126-bp fragment approached the magnitude of the other fragments present, such as peaks at 121-, 210-, 212-, 250-, and 286-bp. A 348-bp fragment was present in every east leg sample analyzed and became the largest peak in T-RFLPs from day 27 and day 30. T-RFLP profiles from well SE1M on day 11, 13, 15, 18 and 21 also displayed similar patterns (data not shown). Unfortunately, there was not a strong correlation between the dominance of any of the major peaks and maximum CAH treatment efficiency. The best treatment occurred between days 4 and 7 where the SE0.5 T-RFLP profiles show a transition from dominance of the 277-bp fragment to the 126-bp fragment. Both the 120-bp fragment and the 348-bp fragment were present throughout the study. As treatment efficiencies decrease from day 7 to day 21, there is not a similar recognizable pattern in the SE0.5 T-RFLP fragments.

Similar to the SE0.5 groundwater results, in the SE1 well, the T-RFLP profile of the groundwater samples beginning on day 11 showed that the bacterial community was dominated by the 126-bp fragment from day 11 to day 21.

Only two samples were obtained from the non-bioaugmented well leg during the study, on days 0 and 21. On day 0, major fragments of 210-, 277-, 279- and 348-bp peaks were observed in a groundwater sample from the SW0.5 well. The 210- and 279-bp fragments accounted for approximately 32% of the total peak area in the west leg T-RLFP profiles at day 0, while they comprised less than 6% of the peak area in samples from the SE0.5 well. By day 21, the community composition in the SW0.5 well had shifted to a more diverse profile with major peaks at 74-, 101-, 117-, 208-, 333-bp, a dominant peak at 348-bp, and many more small peaks. Interestingly, the SW0.5 profile at day 21 is not too dissimilar to the SE0.5 profile at the end of the study. In both cases the 348-bp peak is the dominant

peak, but additional major peaks at 74-, 101-, and 117-bp appear in the non-bioaugmented leg.

Statistical analysis using the GelComparII software was also used to investigate the T-RFLP banding patterns of the groundwater samples taken from wells SE0.5 and SW0.5. A similarity analysis using UPGMA and the Dice coefficient method indicated there were three major clusters exhibiting similar T-RFLP patterns (Figure 4.14). Most of the samples shared at least 80% similarity except the samples collected at day 11, 15 and 30. Cluster A represents the samples collected from SE0.5 from day 0 to day 7 and corresponds to the period when TCA and DCA transformation is increasing and reaches its maximum efficiency. Cluster B represents samples collected from SE0.5 from day 9 to day 27 as the system gradually lost its CAH transformation ability. The two west leg samples obtained on day 0 and day 21 were clustered in Cluster B and were most similar to each other (~85% identity). It was also shown that the groundwater samples of the west leg shared approximately 78% similarity with those from the east leg on day 0 and again on day 21. The community in SE0.5 developed a very unique structure at day 30, exhibiting only 64% similarity with the rest of the samples.

T-RFLP analysis was also conducted with the *Hin6*I restriction enzymes (data not shown). It was found that peaks at 62- and 65-bp from *Hin6*I digests corresponded to 126- and 277-bp peaks from *Mnl*I digests. However, no definitive counterpart to the 120-bp peak from *Mnl*I digestion was observed in the T-RFLP from *Hin6*I digestion as it was not a mono-dominant peak in *Mnl*I-digest T-RFLPs. Unfortunately, none of the bacteria strains showing major peaks in *Mnl*I-digested T-RFLP profiles could be identified using in-silico digestion analysis (TAP T-RFLP, RDP II; available at http://rdp.cme.msu.edu/html/TAP-trflp.html#program for free).

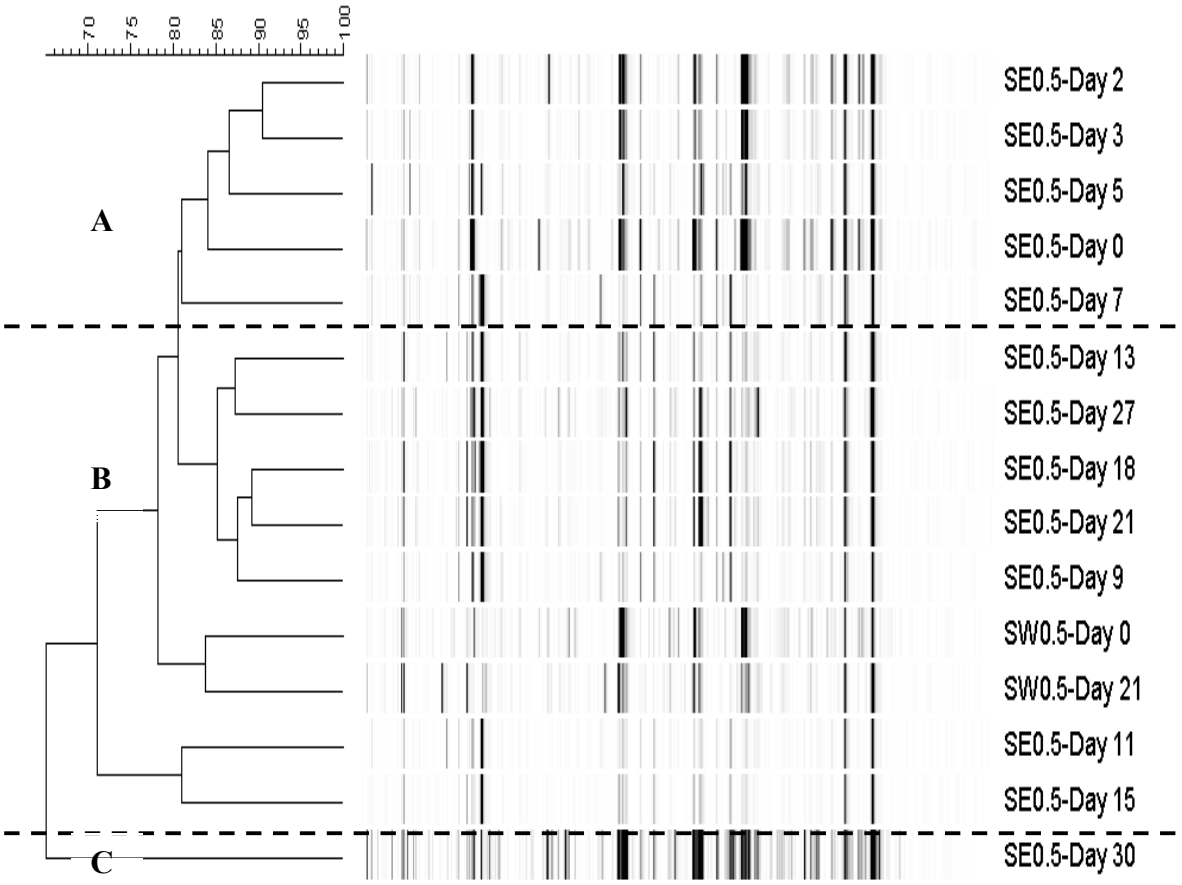


Figure 4.14 A GelComparII-generated UPGMA clustering dendrogram and corresponding normalized restriction profiles from the community DNA extracted from the bioaugmentation experiment samples in well SE0.5.

4.4 Discussion

During this test, effective treatment of chlorinated ethanes was achieved. The maximum removal efficiencies for 1,1,1-TCA and 1,1-DCA were approximately 80% and 96% of the influent values, respectively. In the bioaugmented east zone, butane was depleted in the first three days. During this period, increases in the concentrations of chlorinated ethanes were observed in the east monitoring wells. Since butane was present in abundance for organisms to use as primary growth substrate, chlorinated ethane biotransformation was inhibited. The maximum butane concentrations in wells SE2 and SE3 was approximately 500 µg/L; too little to support growth of a thriving butane-utilizing microbial population. After the 3-day lag period, significant removal of 1,1,1-TCA and 1,1-DCA was observed as butane was reduced to near zero values likelt resulting in less CAH transformation inhibition. Upon correcting for dilution based upon bromide tracer test, the concentration of chlorinated ethanes in all of the east leg monitoring wells was approximately the same; indicating no significant removal occurred past the first monitoring well.

Many factors may be associated with the loss of treatment during the late stage of the bioaugmentation test. The gradual loss of treatment efficiency began around day 6, which corresponds to the time required to grow a butane-utilizing microbial population in the indigenous well leg to sufficient density to utilize the added butane. Transformation product toxicity is common during cometabolism of CAHs and may have had a selective negative impact on butane-utilizing organisms capable of 1,1,1-TCA and 1,1-DCA cometabolism. Estimated transformation capacity values for the bioaugmentation culture were 0.11 mg TCA/mg cells and 0.20 mg DCA/mg cells based on previous microcosm tests and mathematical modeling (Mathias, 2002), which, when combined with a microbial yield, become transformation yields of 0.09 mg TCA/mg butane and 0.16 mg DCA/mg butane.

Successful enumeration of one of the bioaugmented butane-utilizing, CAH co-metabolizing microorganisms, strain 183BP, was achieved by means of 183BP-specific primers using real-time SYBR Green I PCR. Two primers, Ran191F and Ran443R, were designed to be specific to strain 183BP. Specificity analysis indicated that the primer pair has a complete match to sequences of six other known bacterial strains. Melting curve analysis of real time PCR amplicons indicated that only one out of all of the samples generated identifiably significant non-specific amplicons; however, the six known organisms also targeted with the primers would result in amplicons identical to that of strain 183BP. This assay allowed detection and quantification of strain 183BP in groundwater samples collected from the bioaugmentation test site at Moffett, CA.

The real-time PCR cell quantification method is subject to uncertainties due to specificity of primers, rDNA content of the target cells, DNA extraction efficiencies, and inhibition of PCR reactions by unknown sample constituents. In this study, cell quantification was performed by correlating threshold cycle values from SYBR-Green-I-bound 16S rDNA amplicons to sample cell densities. This conversion was based upon two assumptions: 1) each cell contains the same amount of rDNA under all environmental conditions; and 2), DNA extraction efficiency is the same for all sample types over a wide range of cell concentrations. However, since a number of environmental conditions such as nutrient limitation and chemical stress can affect cells' physiological state, the DNA content may vary among cells. Another challenge was the presence of unknown PCR inhibitors. Many of the PCR reactions using non-diluted template DNA did not amplify well. Comparison of non-inhibited undiluted sample amplification to results obtained with 1:10 and 1:100 dilutions showed good agreement, so 1:10 dilutions were used for sample analysis to minimize the effects of PCR inhibition.

A real-time PCR standard curve was performed on serial dilutions of DNA extracted from a pure strain 183BP culture and compared to a standard curve performed on DNA extracted separately from serial dilutions of the culture

covering 7 orders of magnitude. Similar standard curves were produced with cell numbers predicted by DNA dilution being larger than those predicted by separate extractions for threshold cycle values less than 34. For cell counts from 1.5×10^3 cells/ml to 2.5×10^4 cells/ml the two standards produced less than 10% difference in cell estimates. For all threshold cycle values, the difference in cell estimates using the two standards was less than 50%, or one-half an order of magnitude. The method of DNA dilution was the chosen standard used for analysis in this study.

Groundwater samples analyzed by real-time PCR showed that strain 183BP concentration in well SE0.5 reached highest abundance of $(1.46 \pm 0.29) \times 10^5$ cells/ml at the first sample period, 2 days after bioaugmentation. Using a nominal cell weight of 2.8×10^{-13} g dry wt./cell (Rittman and McCarty, 2001), the maximum cell density translates to a mass concentration on the order of 0.04 mg cells/L, a population too small to provide the CAH treatment seen at the field site. Since butane was completely removed by the first monitoring well, microbial growth also preceded the first monitoring well. So, although the waterborne concentrations of strain 183BP were below a density required for effective treatment, there may have been significantly more cells attached to aquifer solids closer to the injection well providing a measure of treatment. The average total cell count was about 3×10^6 cells/ml, or using the same average weight per cell, approximately 0.8 mg/L, which is also low considering a time-averaged butane concentration of approximately 4 mg/L, also indicating that the majority of cells were attached to aquifer solids rather than suspended in the groundwater flow.

Since the 183BP-specific primers perfectly match the sequences of six other known organisms, melting temperatures of the 16S rDNA fragment sequences that can be amplified by these two primers were examined. It was determined from sequence information obtained from GenBank that all of the fragments were expected to have the same length and G+C content. The melting temperatures were calculated using the equation as follows (Stahl and Amann, 1991): $T_d = 4N_{G+C} + 2N_{A+T}$, where N_{G+C} and N_{A+T} are the numbers of G and C bases

and of A and T bases, respectively. The predicted melting temperatures for all fragments were 88 ºC, very close to the value (~88.3 ºC) observed from melting curve analysis. Thus, the melting curve analysis may distinguish non-target amplicons having different melting temperature, but not those originating from any sequence of the six known strains.

No treatment of the chlorinated ethanes was observed in the non-bioaugmented well leg, confirming the inability of the indigenous population to transform 1,1,1-TCA or 1,1-DCA. Additionally, the time to complete butane utilization was more than twice as long as that in the bioaugmented east zone, indicating a small existing population of butane-utilizers prior to chemical augmentation. T-RFLP and real-time PCR results of samples taken from the non-bioaugmented well leg before bioaugmentation and again after a robust butane-utilizing consortia had developed at day 21 showed no evidence of strain 183BP cells in the microbial population of the west well leg.

The T-RFLP pattern indicated that shifts in the native bacterial community structure likely resulted from bioaugmentation and chemical amendment. Although the community remained moderately conserved during the experiment, an organism(s) with a T-RFL of 277-bp that was present in some abundance before bioaugmentation and chemical amendment dominated the community profile for the first three days. Over the next 4 days, the microbial community transitioned to dominance by an organism(s) with a T-RFL of 126-bp that lasted through day 27 of the study (the end of butane addition). During this time, treatment efficiency initially increased and then decreased significantly from days 5-21. An organism with a T-RFL of 277-bp was evidenced in the non-bioaugmented west well leg, indicating that the organism was probably not active towards 1,1,1-TCA. An organism with a T-RFL of 126-bp was not seen in the west well leg samples. Organisms with T-RFLs of 333- and 348-bp were present throughout the entire bioaugmentation test and seen in samples from both well legs and an organism with a T-RFL of 120-bp was present throughout the test in the east

well leg. Unfortunately, none of the T-RFLs could be associated with a phylogenetic type using the RDPII 16S rDNA database and computer-simulated digestion analysis. A highly diverse community structure was observed at day 30 after the termination of butane addition to the field, presumably as part of a transition back to native environmental conditions.

CHAPTER 5
MOFFETT BIOAUGMENTATION TEST (DEC 2003-JAN 2004)

5.1 Introduction

Groundwater contamination is of significant health concern since it is used as drinking water supply by many people. 1,1,1-Trichloroethane (1,1,1-TCA) is a man-made halogenated solvent and primarily used as industrial cleaning and degreasing agent. It is one of the common CAH contaminants found in groundwater and soil environments. The maximum contaminant level (MCL) and maximum contaminant level goal (MCLG) of 1,1,1-TCA in drinking water are 0.2 mg/L (EPA, 2002). Bacterial cultures isolated from various environmental media are capable of degrading 1,1,1-TCA either through aerobic cometabolic or anaerobic pathways (Egli et al, 1987; Galli and McCarty, 1989; de Best et al 1999; Yagi et al., 1999; Kim et al, 1997).

The Moffett Federal Airfield In-Situ Bioremediation Test Site (Mountain View, CA) is one of the sites that were contaminated with various chlorinated aliphatic hydrocarbons (CAH), including 1,1,1-TCA. Remediation assessment studies conducted at Moffett field showed that methane-oxidizing bacteria which use methane as primary substrate and cometabolically transform CAHs with a methane monooxygenase enzyme achieved significant transformation of vinyl chloride (VC) and trans-1,2-dichlroethene (t-DCE), but exhibited limited transformation of 1,1,1-TCA, trichloroethylene (TCE) and cis-1,2-dichlroethene (c-DCE) (Semprini et al., 1990; Semprini et al., 1991). In other field tests (Hopkins et al., 1993; Hopkins and McCarty, 1995), equally effective removal of c-DCE and TCE by phenol- and toluene-oxidizing bacteria whose toluene oxygenase enzyme is responsible for CAHs cometabolism were observed, but t-DCE was least transformed. However, in microcosm studies none of microcosms effectively degraded 1,1,1-TCA when fed phenol, toluene, methane or ammonia (Hopkins et

al, 1993; Hopkins and McCarty, 1995). Fries et al (1997) identified 63 strains from 273 phenol- and toluene-degrading isolates that grew in a Moffett Field, CA., aquifer and found that most of them can cometabolize TCE , which indicates that there a significant number of naturally occurring strains may support successful phenol- and toluene-stimulated TCE transformation.

Bioaugmenting microorganisms with known degradative abilities may improve biotransformation of chlorinated aliphatics at Moffett Field. Several bioaugmentation treatment tests conducted at contaminated sites resulted in successful transformation the targeted toxins to harmless compounds (Baud-Grasset et al, 1995; Fantroussi et al, 1999; Major et al, 2002; Salanitor et al, 2000). Kim et al (1997) enriched a mixed community from a CAH-contaminated DOE site in Hanford, WA. The enrichment culture was able to cometabolize 1,1,1-TCA, 1,1-DCE and a number of other CAHs using butane as a primary substrate. A butane-utilizing organism was isolated from the enrichment and was shown to have 1,1,1-TCA, 1,1-DCA, and 1,1-DCE transformation abilities (Mathias, 2001). Characterization of the 16S rRNA gene of the organism was found 100% identical to a known *Rhodococcus sp*. USAN-12 (Genbank accession number AF420413). During the past few years, a number of field bioaugmentation pilot tests with cultures containing strain 183BP have been conducted at the Moffett field. The ability of the inocula to survive, adapt and flourish in a non-native environment and function well in biodegradating the targeted contaminants is an important concern in bioaugmentation.

Microbial analysis may provide a critical key in understanding the contaminant biodegradation process. Traditional approaches like culture plating and the most probable number (MPN) method are often too selective, time-consuming and inaccurate. The development of molecular-based techniques allows detecting and/or quantifying microorganisms in different environments using 16S rDNA/rRNA genes (Hugenholz et al, 1998; Hendrickson et al 2002; Dojka et al,

1998; Fennell et al 2001; Major et al, 2002; Löeffler et al., 2000; DeLong et al., 1999).

To date, bioaugmentation treatment tests are always critically assessed in terms of efficiency and effectiveness. In most cases, the inocula are usually highly active and efficient in removal of the contaminants under laboratory conditions; whereas, it is difficult to predict their performance under natural conditions, and very little is known about actual cell densities and spatial distribution over the duration of the bioaugmentation treatment test. Hence, the objective of this research was to develop a quantitative SYBR Green I real-time PCR assay based upon 16S genes to provide assessment of the abundance and role of an augmented culture (stain 183BP) in 1,1,1-TCA biodegradation tests conducted at the Moffett field, CA and to characterize the bacterial community structure and possible community shifts corresponding to 1,1,1-TCA biodegradation using the Terminal Restriction Fragment Length Polymorphism (T-RFLP) method and statistical analysis.

5.2 Materials and methods

In this study, a butane-utilizing mixed culture was bioaugmented to the subsurface at the Moffett Federal Airfield In-Situ Bioremediation Test Site (Moffett Field), Mountain View, CA to cometabolically transform a mixture of 1,1,1-TCA, 1,1-DCA, and 1,1-DCE. The aquifer test zone and operational conditions were the same as those used in the October, 2003, bioaugmentation study detailed in Chapter 4. The test zone consisted of two hydraulically separate, recirculating well legs each containing an injection well, an extraction well, three groundwater monitoring wells spaced 1.0 m, 2.2 m, and 4.0 m from the injection well, and two fully-penetrating wells spaced 0.5 m and 1.5 m from the injection well to hold coupons containing solid support media. The injected groundwater was sparged with either butane or oxygen in separate pulses to provide primary substrate. The CAHs and bromide were added continuously. Community 16S

rDNA was extracted from groundwater and solid coupon samples taken from the monitoring wells and used for real-time PCR and T-RFLP analyses. The 183BP-sepefic primers, Ran191F and Ran44R, were used for real-time PCR reactions; whereas, the universal primers, 27F-B-FAM and 338Rpl, were used for T-RFLP analysis.

5.3 Results

5.3.1 Degradation of chlorinated aliphatics

The concentrations of 1,1,1-TCA, 1,1-DCA, 1,1-DCE, dissolved oxygen (DO), and butane were monitored routinely in all injection and monitoring wells. Six hours prior to bioaugmentation, oxygen-purged groundwater was injected into both well legs. Approximately 5 g dry wt. of a mixed butane-utilizing culture was diluted in 4 L of groundwater and introduced into the injection stream to the east well leg over a period of ten minutes. Immediately after bioaugmentation, chemical amendment of both well legs began. CAHs and bromide were introduced continuously at approximately 150 µg/L for 1,1,1-TCA and 1,1-DCE, about 250µg/L for 1,1-DCA, and 100 mg/L for bromide. Alternate pulses of butane-purged and oxygen-purged groundwater at influent concentrations of about 30 mg/L and 40 mg/L, respectively, were also initiated. Gases were introduced into the injection flow upstream of CAH addition by alternately purging the recycled groundwater with butane and oxygen, with brief nitrogen purges in between to prevent formation of explosive gas mixtures and to limit biomass growth in the injection lines and in the immediate vicinity of the injection well. Purge cycles were 9 minutes of butane purging followed by 4 minutes of nitrogen purging, 156 minutes of oxygen purging, and another 4 minutes of nitrogen purging before repeating the cycle. On day 10, the primary substrate was changed from butane to propane. Propane was added in a manner similar to butane, using the same cycle times. On day 15, primary substrate addition was stopped, but dissolved oxygen, CAH, and bromide addition continued.

Unfortunately, since the study was conducted in the lowlands near San Francisco Bay in the rainy season, hydraulic control of the flowfield was transiently compromised a few times during the study. These events can be observed in the bromide breakthrough data for the two well legs. In the bioaugmented east well leg, bromide breakthrough at the third monitoring well, SE3, was significantly affected by changes in the regional groundwater flow. On days 5, 9, and 12 bromide capture in SE3 dropped from its normal value of around 90% to lows of 68%, 45%, and 56%, respectively. There appears to be full recovery of hydraulic control at SE3 around day 8 before the second and third events ensue, resulting in diminished capture in SE3 from day 9 through day 13. Capture in wells SE1 and SE2 was unaffected on day 5, remaining at 97% or greater, but dropped slightly to approximately 87% and 83%, respectively, around days 9.5 and 12.5. Similarly on the west well leg, capture at SW3 was significantly affected around days 5, 10, and 13. Additionally, there were increases in bromide capture at SW2 on days 10 and 13, when the capture at SW3 decreased, indicating significant changes in the regional flow at these times. Capture at SW3 reached lows of 74%, 66%, and 46% on days 5, 9, and 13, respectively, and was significantly lower than its normal 87% recovery on days 4-6, 9-11, and 12-14. Capture at SW1 was also affected around days 5 and 9, but to a lesser extent. These deviations in recovery efficiency should be taken into account when interpreting chemical and microbiological data from the field test.

Times to 50% breakthrough of bromide in the east well leg were approximately 0.6 d, 0.9 d, and 1.8 d for wells SE1, SE2, and SE3, respectively. In the west well leg, 50% breakthrough occurred at approximately 0.15 d and 0.8 d at wells SW1 and SW3. These breakthrough values are similar to those obtained from the October test (Ch.4).

At monitoring wells SE1 and SW1, butane concentrations did not decrease to below detection during the 10 days of butane addition as they did during the October test. The butane concentrations at well SW1 (~4000 μg/L) were

nearly twice those of well SE1 (~2000 μg/L) prior to enhanced utilization from day 4 to day 10 (Figure 5.2). Butane concentrations in well SE2 and SE3 were significantly lower than in well SE1 indicating that a butane-utilizing microbial community was present past the first monitoring well. However, no significant difference in butane concentration was observed in the west well leg between wells SW1 and SW3. In contrast to the current bioaugmentation test, it took only 3 days to achieve complete butane removal in the bioaugmented east leg during the October test. Toxicity caused by 1,1-DCE transformation products may have inhibited microbial growth in the bioaugmented east leg during the current test. On day 10, butane addition was stopped and the primary substrate was switched to propane.

Unlike the October test, the oxygen utilization rate in the east leg was fairly low (Figure 5.3). The oxygen concentration in well SE1 reached a stable level of 30 mg/L after breakthrough, maintaining only about 10 mg/L oxygen removal between the injection well and the first monitoring well. There appeared to be about 5 mg/L oxygen utilization in the east leg between the first and second monitoring wells, which is consistent with the observed butane profile. Although the dissolved oxygen concentration in the west injection well was slightly higher than in the east, concentrations in wells SW1 and SW3 were similar to well those in SE1, with no oxygen removal observed beyond SW1.

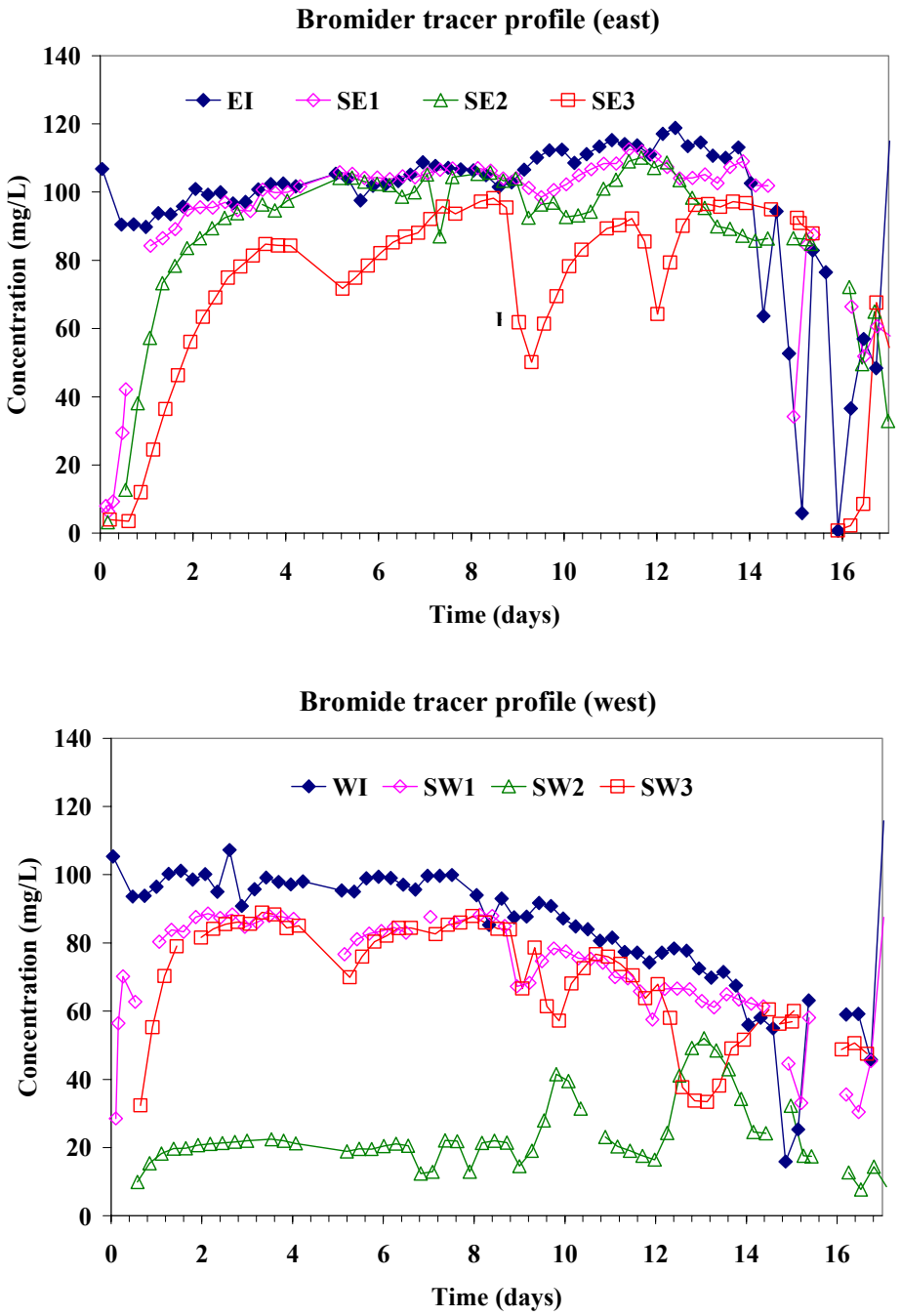


Figure 5.1 Bromide tracer concentrations in the injection wells and the monitoring wells in the two test zones (east, bioaugmented; west, non-bioaugmented) at Moffett Field (data from Gary Hopkins at Stanford University, CA)

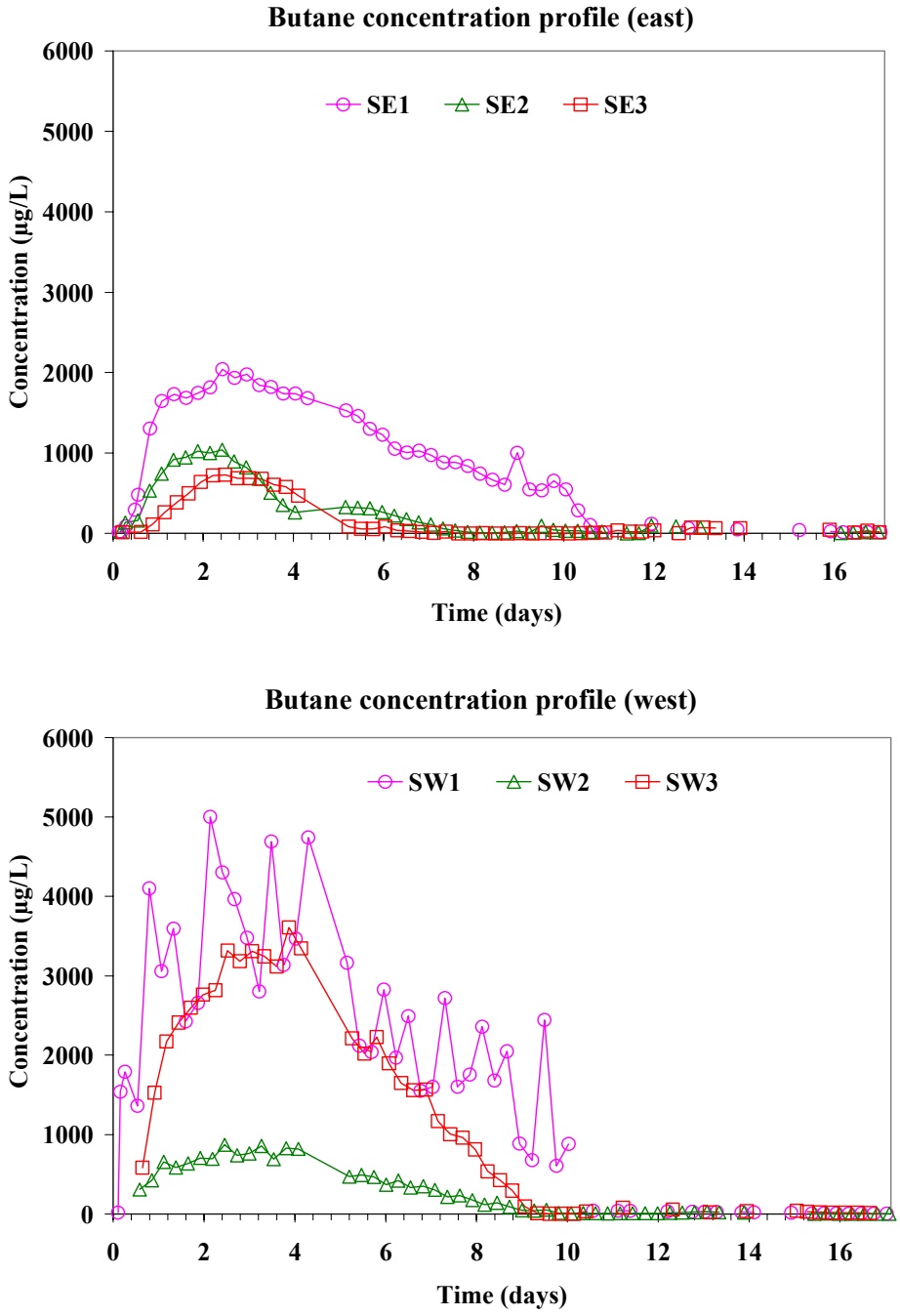


Figure 5.2 Butane concentrations in the injection wells and the monitoring wells in the two test zones (east, bioaugmented; west, non-bioaugmented) at Moffett Field (data from Gary Hopkins at Stanford University, CA)

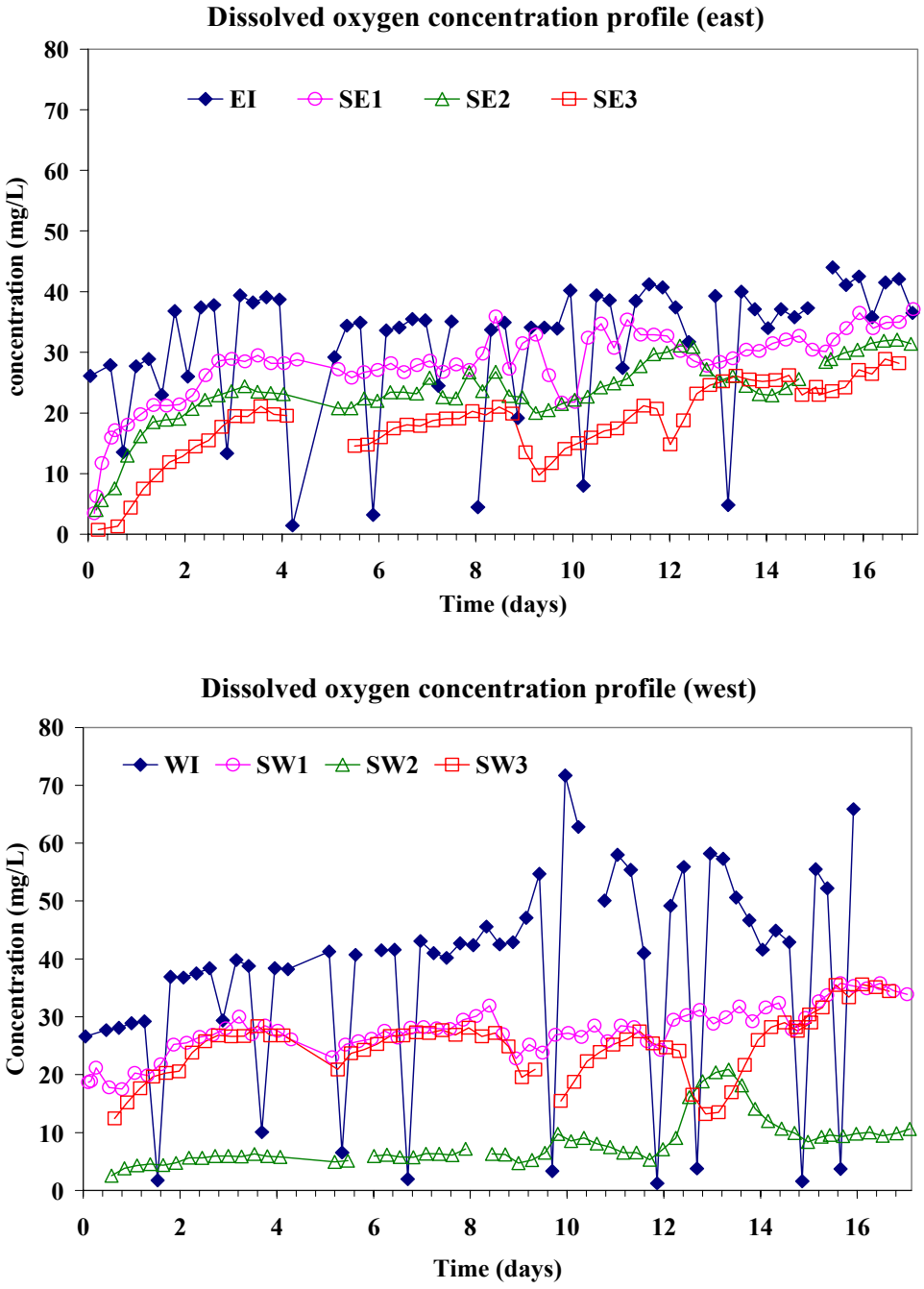


Figure 5.3 Concentrations of dissolved oxygen in the injection wells and the monitoring wells in the two test zones (east, bioaugmented; west, non-bioaugmented) at Moffett Field (data from Gary Hopkins at Stanford University, CA)

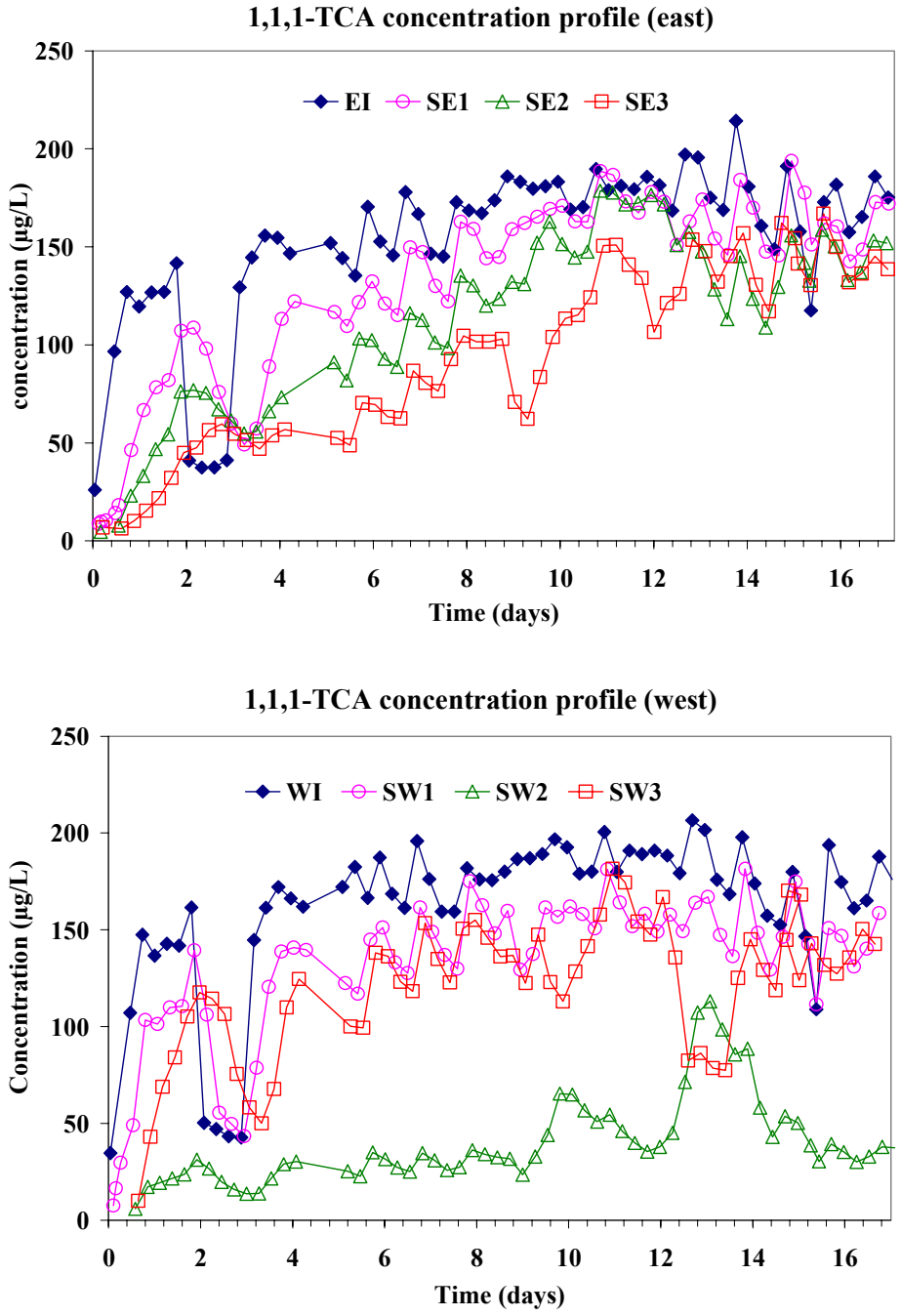


Figure 5.4 1,1,1-TCA concentrations in the injection wells and the monitoring wells in the two test zones (east, bioaugmented; west, non-bioaugmented) at Moffett Field (data from Gary Hopkins at Stanford University, CA)

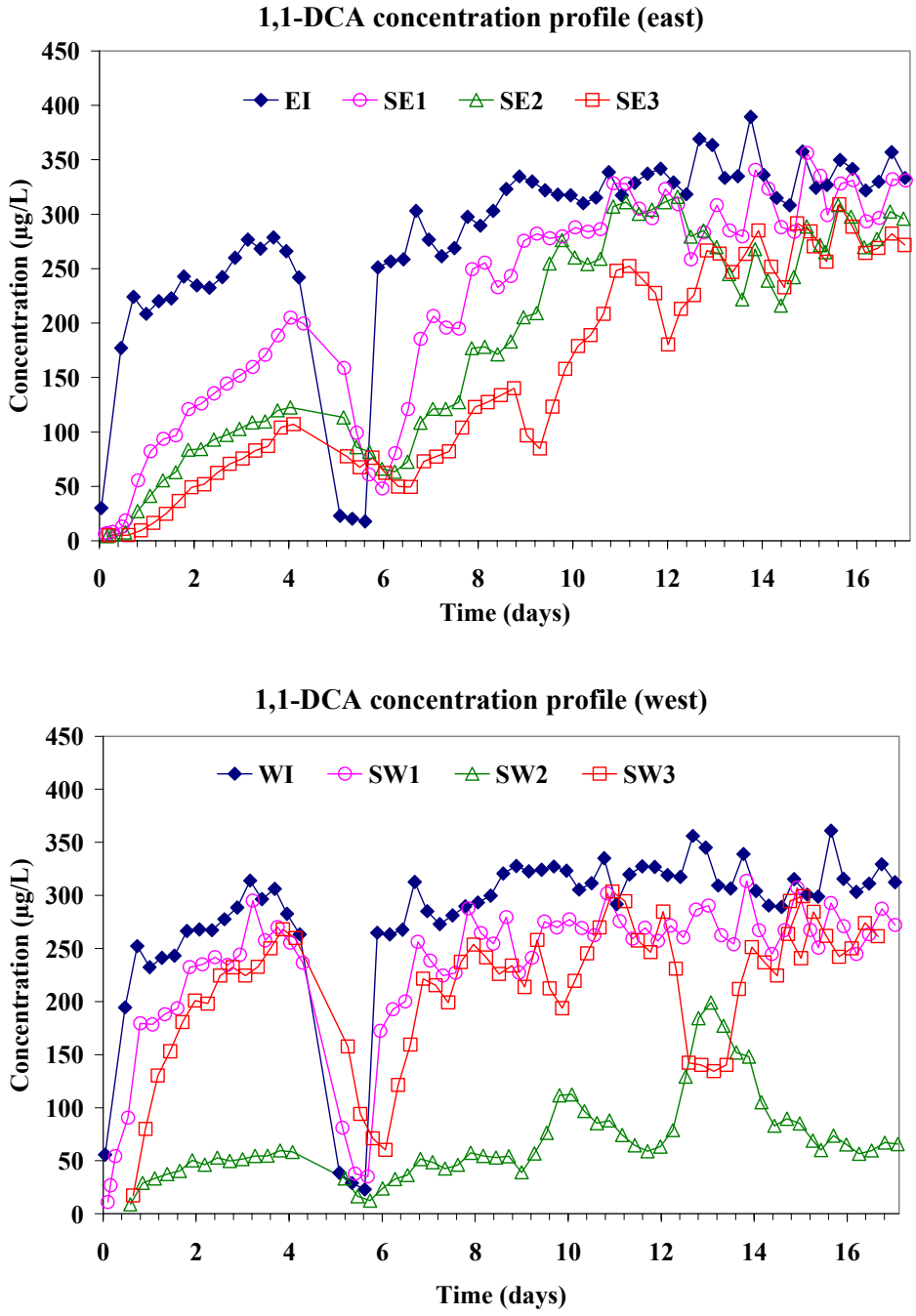


Figure 5.5 1,1-DCA concentrations in the injection wells and the monitoring wells in the two test zones (east, bioaugmented; west, non-bioaugmented) at Moffett Field (data from Gary Hopkins at Stanford University, CA)

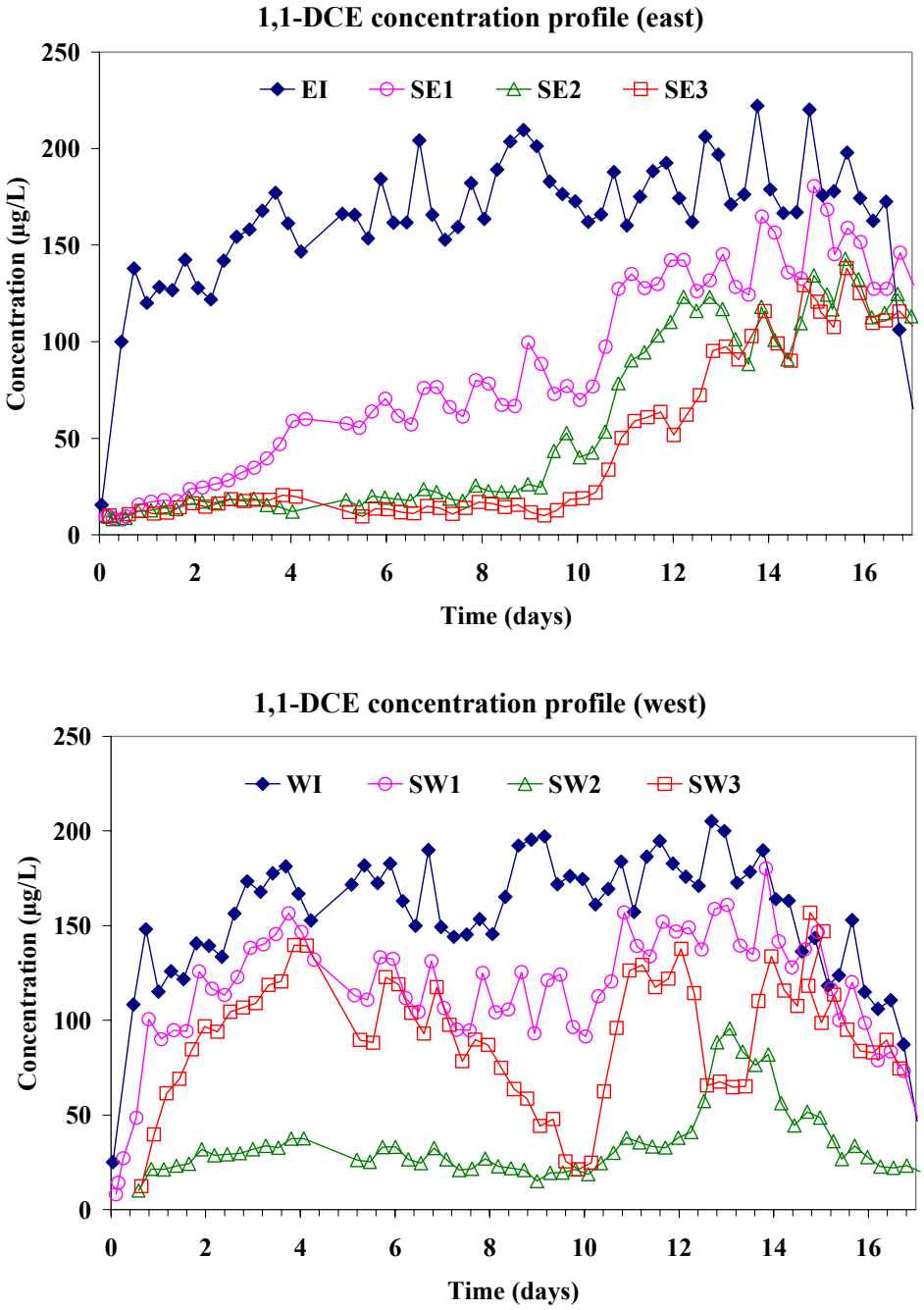


Figure 5.6 1,1-DCE concentrations in the injection wells and the monitoring wells in the two test zones (east, bioaugmented; west, non-bioaugmented) at Moffett Field (data from Gary Hopkins at Stanford University, CA)

In the non-bioaugmented west leg, 1,1,1-TCA and 1,1-DCA concentrations reached 90% of the influent values within 3days and remained at this level throughout the test (Figure 5.4 and Figure 5.5). The difference in concentrations of chlorinated ethanes between the injection well and the monitoring wells was most likely due to dilution, as indicated by the bromide capture recoveries for these wells. The sharp dip in 1,1,1-TCA injection concentrations from day 2 to 3 and 1,1-DCA concentrations on days 4 to 6 were caused by mechanical failures during field operation. Times to 50% 1,1,1-TCA and 1,1-DCA breakthrough were about 0.8 day for well SW1 and 1.4 day for well SW3. In the bioaugmented east leg, 1,1,1-TCA concentrations gradually increased for the first 2 days followed by a dip in concentration around day 3.5 due to mechanical failures, and then a steady rise to influent concentrations by day 11.

Since butane was still present at a concentration around 1500 μg/L, cells were not in an optimum growth state and 1,1,1-TCA transformation was inhibited during this period. Unlike the October test in the absence of 1,1-DCE contamination, biotransformation of 1,1,1-TCA and 1,1-DCA occurred beyond monitoring well SE1, as well as between the injection well and the first monitoring well.

Figure 5.6 shows 1,1-DCE concentrations in the injection wells and in the monitoring wells during the 17-day operation period. In the east well leg, 1,1-DCE transformation was observed out to the second monitoring well. Lower butane concentrations past the first monitoring well likely resulted in less inhibition of 1,1-DCE transformation. With the 1,1-DCE injection concentration at approximately 175 μg/L, approximately 93% reduction in the influent 1,1-DCE was observed during the first 10 days of the study. When the primary substrate was changed from butane to propane on day 10, 1,1-DCE concentrations increased throughout the test zone.

In the non-bioaugmented west zone, 1,1-DCE was injected at the same levels as that of the east zone. In wells SW1 and SW3, 1,1-DCE concentrations

approached influent values within the first 4 days of the test. Approximately 25-35% 1,1-DCE removal was observed in the first monitoring well between days 4 and 10. The third monitoring well showed similar removal through day 8, but concentrations decreased to 20% of the injection concentrations by day 10, followed by a rapid rebound to concentrations roughly equivalent to those in SW1, with a similar dip occurring around day 13. It is difficult to determine if the DCE concentration drop in SW3 was the result of transformation or due to flow events. The increase in removal efficiency occurred at a time when the butane concentrations decreased to near zero values in SW3 (around day 8 to day 10), possibly resulting in less butane inhibition of DCE transformation. However, there was a flow event around day 9-10 that impacted other chemical concentration profiles, but not to the extent seen in the DCE concentrations for well SW3. Also, on day 13, the 1,1-DCE concentration in well SW2 rises significantly, consistent with the concentration profiles of other chemicals indicating that a change in the regional flow field occurred. A similar increase in 1,1-DCE concentrations in SW2 did not occur around day 9-10 during the period of increased DCE removal, suggesting that transformation may have been occurring. DCE removal dropped quickly after the switch to propane as a primary substrate, with influent values being reached at approximately day 11 and again on day 14.

5.3.2 Detection and quantitation of strain 183BP during bioaugmentation

A T-RFLP analysis using the restriction endonuclease MnlI showed four major peaks at 47-, 174, 183-, and 254-bp in community DNA extracted from the bioaugmentation culture (Figure 5.8, culture). The 183-bp peak accounted for 13.5% of the total peak area in the T-RFLP profile.

Groundwater sampling for microbial analysis was conducted at all of the east monitoring wells (SE1, SE2, and SE3) as well as the fully penetration wells SE0.5, SE1.5 and SW0.5 (Figure 5.7). Prior to bioaugmentation, background samples taken from the east well legs revealed that strain 183BP was present at a

density ranging from 10 and 500 cells/ml. These trace background levels were consistent with residuals found after previous bioaugmentation tests conducted at the site. In samples from well SE0.5, a greater than 2-log-order increase in strain 183BP cell concentrations was observed 0.4 day after bioaugmentation. After day 0.4, the cell concentration dropped to approximately 10,000 cells/ml and remained at this level through day 10. During this period, effective treatment of 1,1-DCE was achieved in the bioaugmented east zone. Higher cell concentrations were seen on days 15 and 17 after butane addition was terminated, which may have been the result of cell sloughing due to a switch in primary substrate, or due to the cessation of primary substrate addition on day 15. The time to achieve the maximum cell concentration in wells SE1 and SE2 lagged that to well SE0.5 by about 0.3 days. Cell concentrations seen in wells SE1 and SE2 were also lower than those seen in SE0.5. Although further from the injection well, well SE2 had about a 1-log-order higher cell concentration than the fully penetrating well SE1.5. The densities of strain 183BP in well SE1.5 were below 600 cells/ml over the entire test and showed high variability, as these concentration levels were equal to the lowest concentration in the calibration curve. This may have been due in part to sampling procedures used during the test that resulted in flushing of the SE2 well during the acquisition of groundwater for chemical analyses at least once per day, while this flushing did not occur for well SE1.5. No positive real-time PCR signals were detected from samples collected from the SE3 well, indicating very few or no 183BP cells were transported that far or grew at this location. In the non-bioaugmented west zone, a sample taken from well SW0.5 on day 16.9 resulted in a positive signal with a calculated density of 19.4 cells/ml, below the quantifiable detection limit.

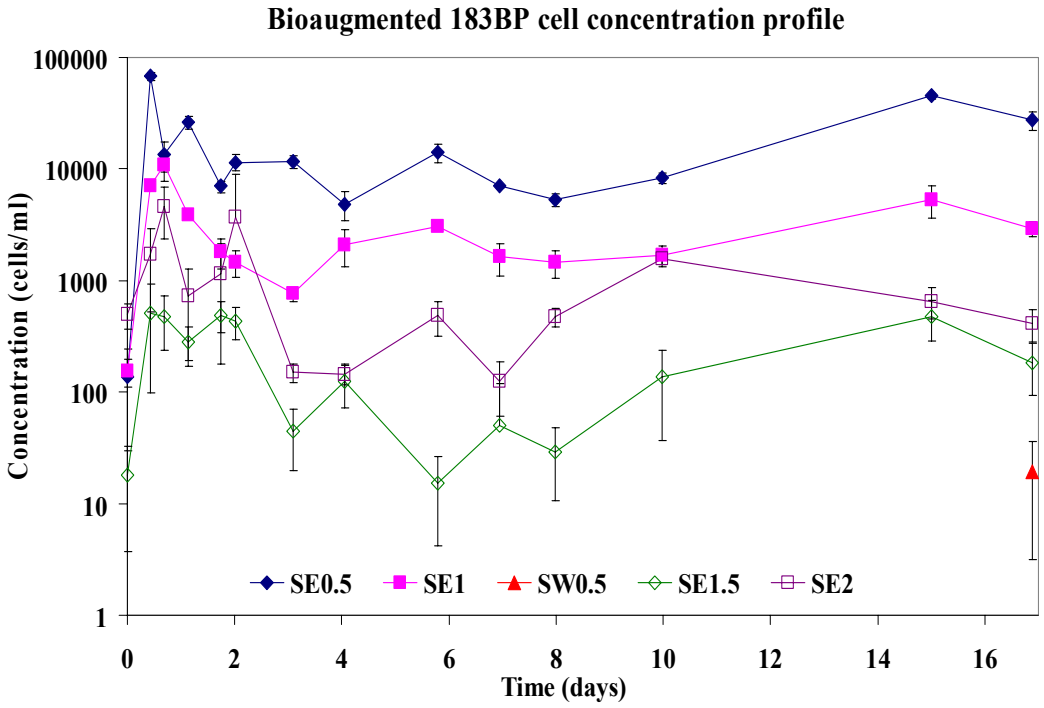


Figure 5.7 Cell concentrations of strain 183BP in the monitoring wells (SE0.5, SE1, SE1.5, SE2, and SW0.5) at Moffett Field (No fluorescent signals were detected from samples taken from well SE3).

5.3.3 Community T-RFLP profiles

Figure 5.8 shows the T-RFLP profiles of the bioaugmentation culture and the community 16S rDNA in the monitoring well 0.5m from the injection well using universal bacterial primers (27F-B-FAM and 338Rpl) and *Mnl*I restriction enzyme. The T-RFLP profile of the bioaugmentation culture contained four major peaks at 47-, 174-, 183- and 254-bp; however, the same peaks accounted for less than 10% of the total peak area any T-RFLP profile obtained from field samples at any time during the study. Similar to the results from the October test, T-RFLP analysis of groundwater samples showed no significant levels of 183-bp fragments in any of the samples. Also similar to the October test, an early stage dominance of

the T-RFPL profiles by a fragment of 277-bp transitions to dominance by a 126-bp fragment after about five days of operation.

Before bioaugmentation, peaks at 120-, 277- and 348-bp dominated in the groundwater bacterial community. Immediately after bioaugmentation and the onset of chemical amendments, a reasonably large peak at 47-bp occurs in all of the east leg samples. However, the relative size of the 47-bp peak diminishes quickly as fragments of 277-bp dominate the profile through day 2, accounting for at least 40% of the total peak area; whereas, the peaks at 120- and 348-bp also diminished relative to the 277-bp peak (Figure 5.9). The primary community structure changed markedly from day 0 to day 0.4, but remained relatively unchanged from day 0 through day 2 when butane and DO were present in relatively high concentrations. At day 3.1, a more diverse microbial community appears to have developed or, since the analysis is relative to the largest peak present, perhaps the amount of 277-bp fragments diminished to that of the other organisms present. A similar community structure was observed on day 4.1, but by day 5.8 a distinctly different community structure emerged with a 126-bp fragment dominating the groundwater microbial community. The maximum percentage of the 126-bp peak occurred on day 7. By day 10 the 126-bp peak appears to be shrinking relative to the other peaks and by day 15 and day 16.9, again a relatively diverse population emerges.

The growth substrate was changed from butane to propane on day 10 and terminated completely on day 15; the community changes from day 10 to day 16.9 may reflect that change. A peak arises at 242-bp that was not prominent before the substrate change. On day 16.9, the major peaks present in samples taken from the SE0.5 well and the SW0.5 well were different. Peaks at 71-, 73-, 120-, 126-, 212-, 242-, and 253-bp dominated the groundwater microbial community in well SE0.5. In well SW0.5, dominant peaks occurred at 74-, 241-, 253-, and 277-bp, with only the 253-bp fragment in common with profiles from well SE0.5 on the same day.

Figure 5.10 illustrates changes of the 47-bp fragment peak area in T-RFLP profiles of the groundwater samples. In the bioaugmentation culture, the 47-bp peak dominated the T-RFLP profile of the bioaugmentation culture and accounted for 23% of the total peak area. No T-RFs of 47-bp were detected in groundwater samples taken prior to the bioaugmentation test, except in well SE1.5 where the 47-bp peak accounted for less than 0.7% of the total peak area. In all of the east leg monitoring wells, except SE3, the 47-bp fragment accounted for about 10% of the total peak area of the bacterial community members less than one day after bioaugmentation. The percentage of 47-bp fragments decreased rapidly to around 1% or less through day 8 and then slowly increased to around 1% to 4% at the end of the test.

In order to investigate the similarity of T-RFLP banding patterns from different samples, a cluster analysis using GelComparII software (Applied Maths, Belgium) was performed. Samples from well SE0.5 were grouped into three clusters at a 74% level of similarity using UPGMA and the Dice coefficient method (Fig 5.11). Cluster A contained 7 lanes and represented the samples collected during the initial stage of the bioaugmentation from day 0 to day 4.1, which was dominated by a 277-bp peak. With a similarity level of 90%, cluster B was composed of 4 samples collected from day 5.8 to day 10 when the 126-bp peak dominated the community profile. Samples of day 15 and day 16.9 in well SE0.5 exhibited 91% identity with each other and had about 80% similarity with the sample collected from SW0.5 on day 16.9. The switch of primary substrate feeding from butane to propane on day 10 appeared to stimulate a more diverse bacterial community in the groundwater. Similarity analysis was also performed to compare the bacterial community in the five wells at given time points (Figure 5.12). It was observed that during the first few days, T-RFLP profiles of samples taken from wells SE0.5 and SE1 were grouped together with a minimum of 78% similarity. Over the course of the bioaugmentation test, the five monitoring wells showed a minimum of 70% similarity in community profiles.

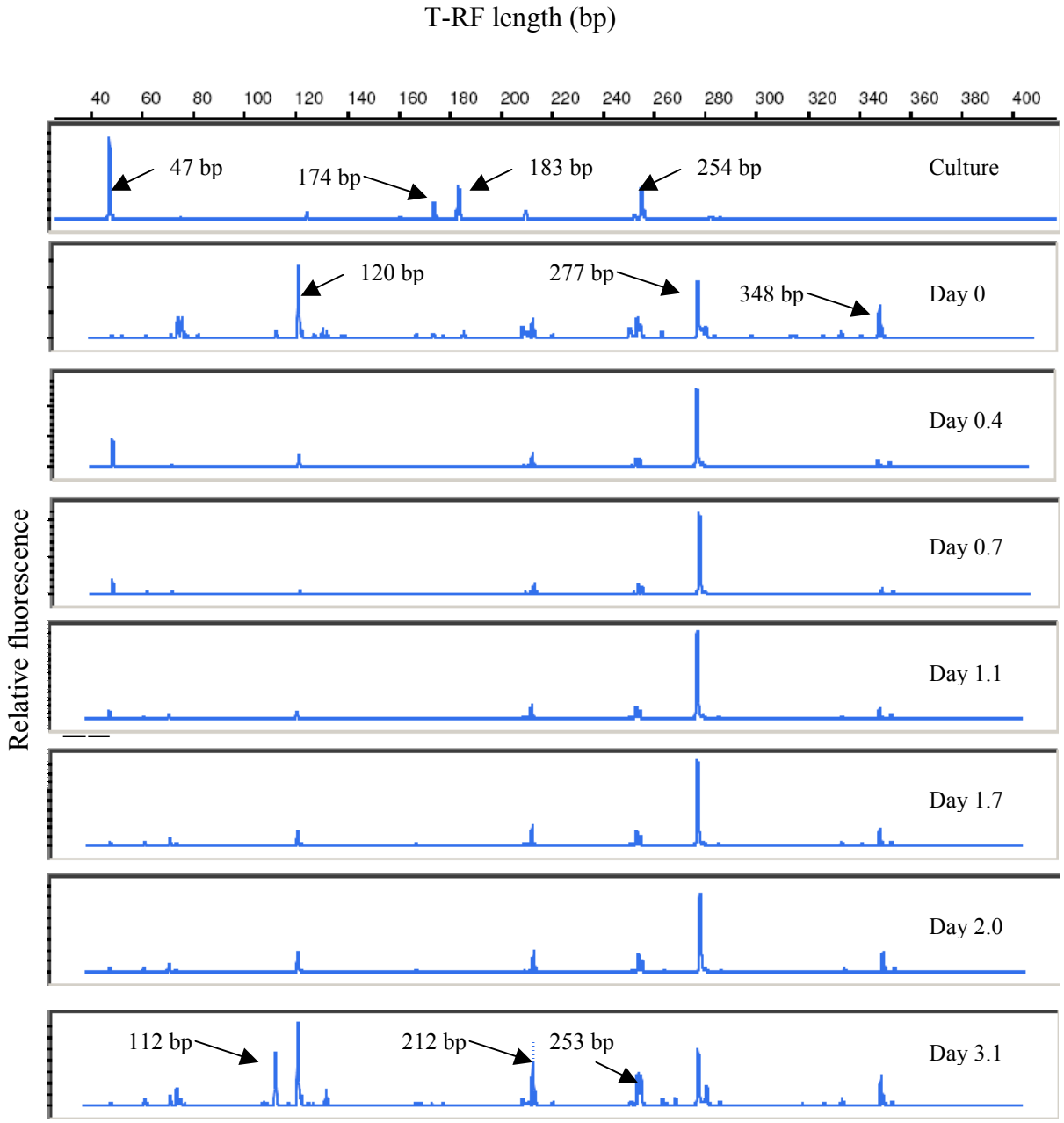


Figure 5.8 T-RFLP profiles generated from the bioaugmentation culture grown in the lab and from groundwater samples taken from well SE0.5 and SW0.5 during the course of the test. Universal bacterial primers (27F-B-FAM and 338Rpl) were used in the PCR reactions and the restrictions were performed with the endonuclease *Mnl*I (Fermentas, Inc).

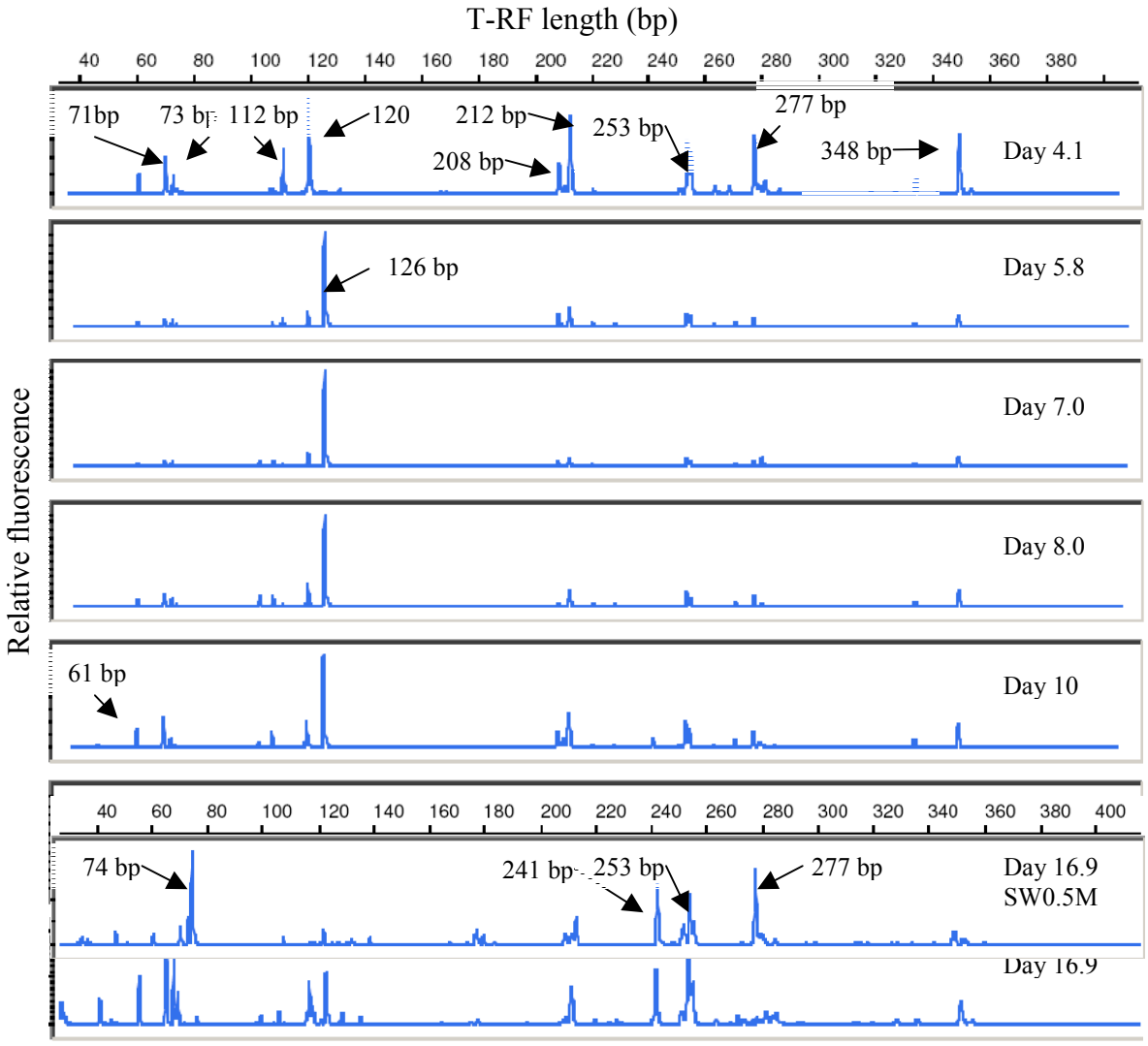


Figure 5.8 (Continued) T-RFLP profiles generated from the bioaugmentation culture grown in the lab and from groundwater samples taken from well SE0.5 and SW0.5 during the course of the test. Universal bacterial primers (27F-B-FAM and 338Rpl) were used in the PCR reactions and the restrictions were performed with the endonuclease *Mnl*I (Fermentas, Inc).

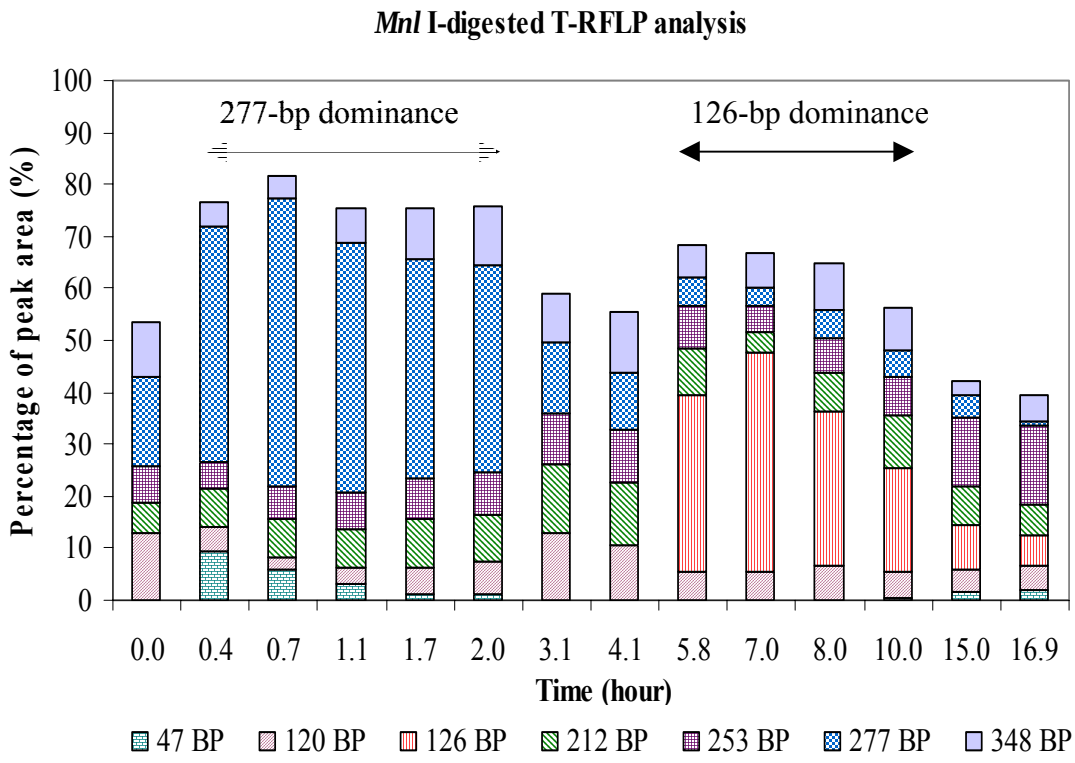


Figure 5.9 Changes in the groundwater bacterial community structure during the bioaugmentation test in well SE0.5.

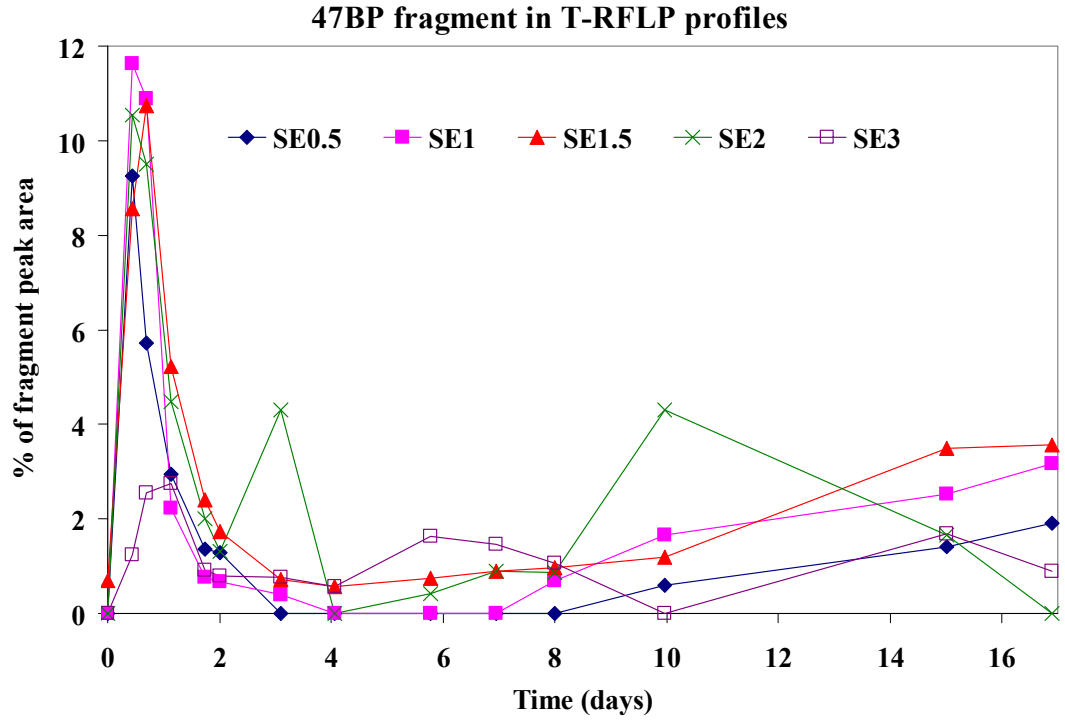


Figure 5.10 The percentages of 47-bp fragment peak areas in T-RFLP profiles from *Mnl*I endonuclease digestion of samples taken from the monitoring wells (SE0.5, SE1, SE1.5, SE2 and SE3) during the duration of the bioaugmentation test.

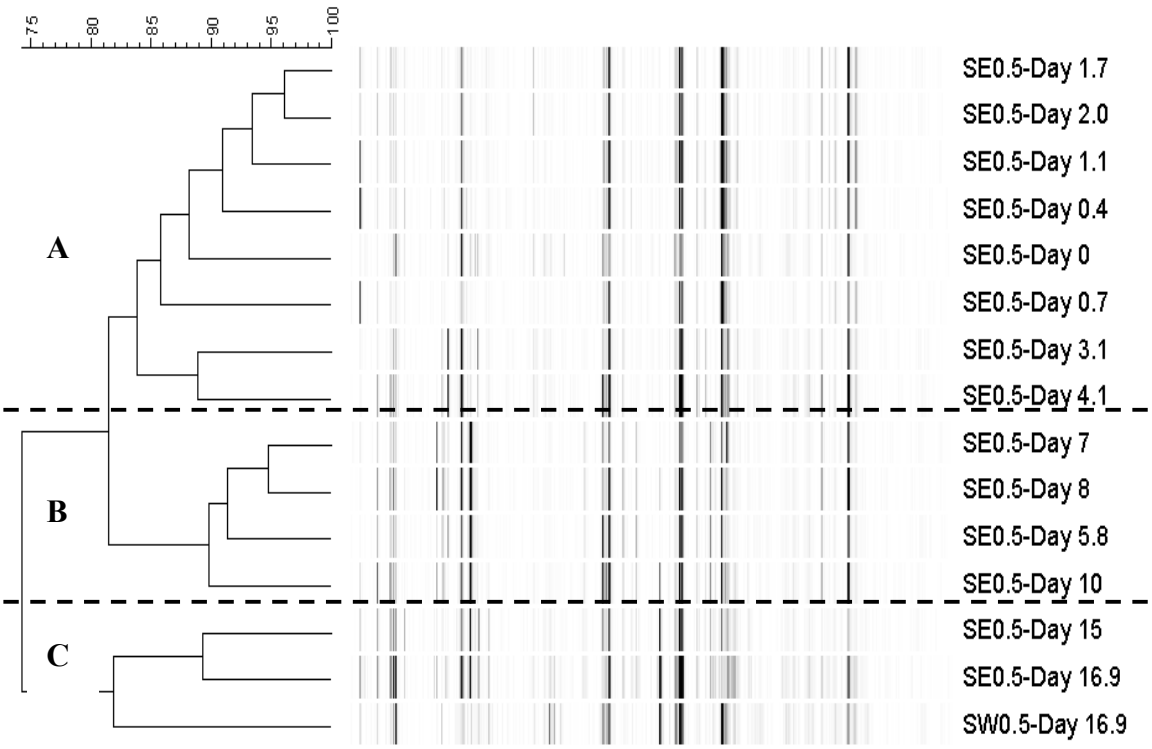
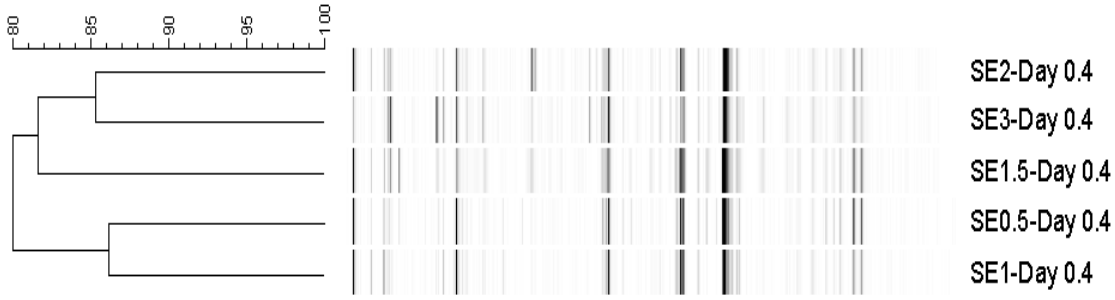


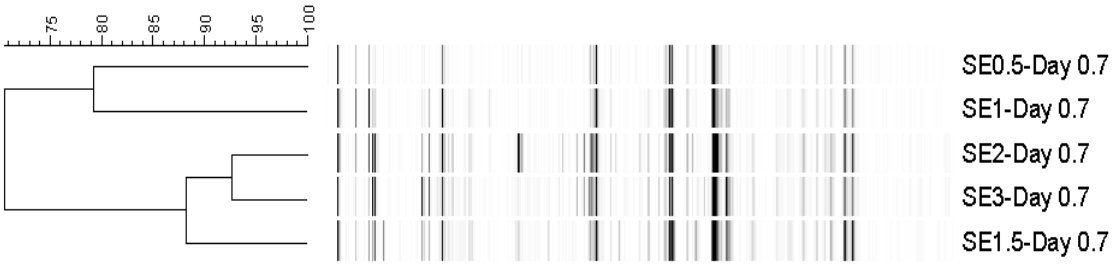
Figure 5.11 A GelCompar II-generated UPGMA clustering dendrogram and corresponding normalized restriction profiles from the community DNA extracted from the bioaugmentation experiment samples in well SE 0.5 and SW0.5.

A. Day 0

B. Day 0.4



C. Day 0.7



D. Day 1.1

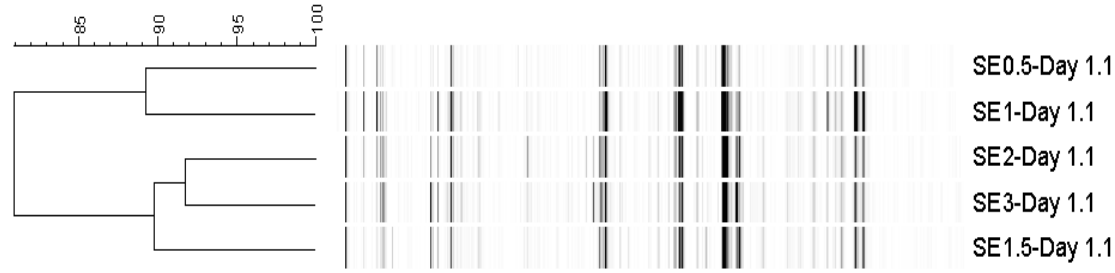
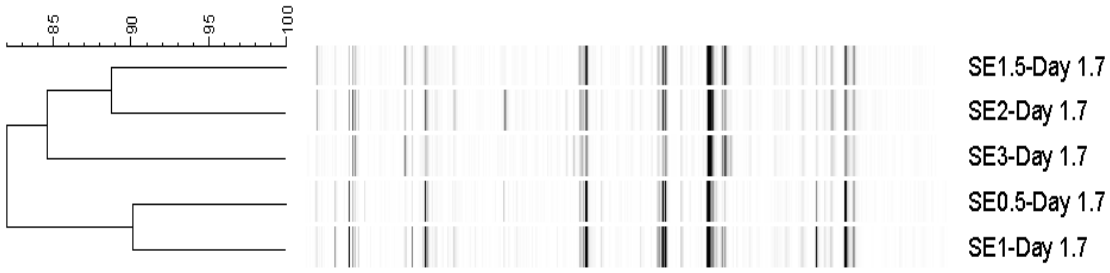
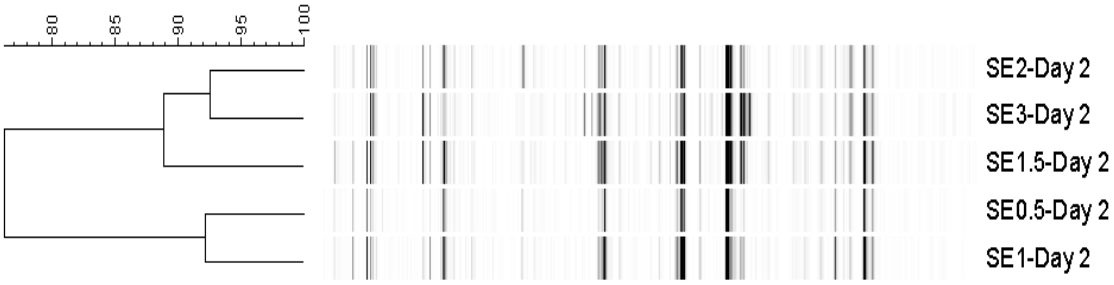


Figure 5.12 A GelCompar II-generated UPGMA clustering dendrogram and corresponding normalized restriction profiles from the community DNA extracted from all the monitoring wells in the bioaugmented zone.

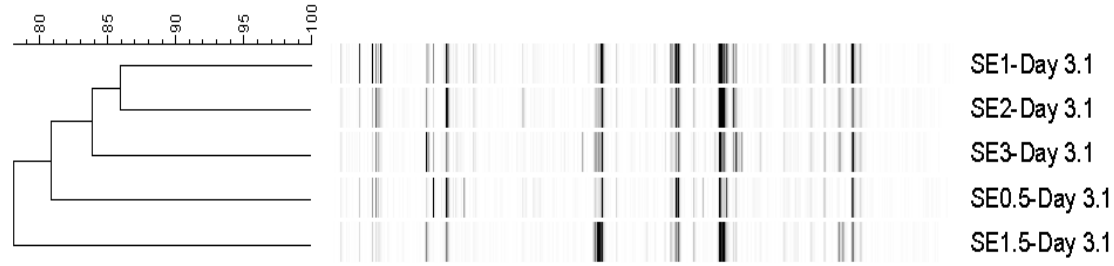
E. Day 1.7



F. Day 2



G. Day 3.1



H. Day 4.1

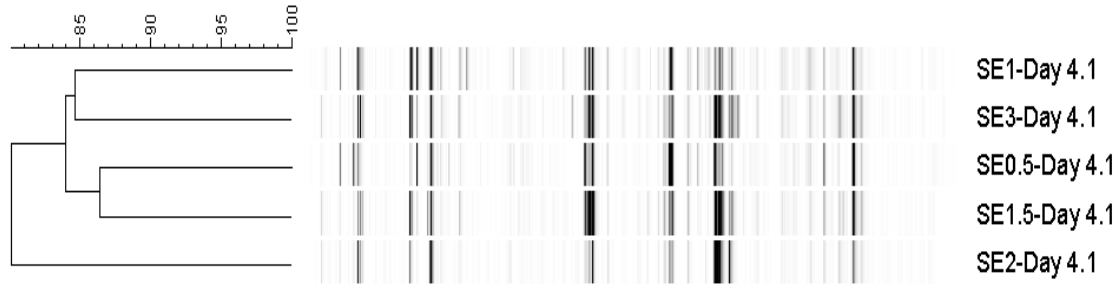
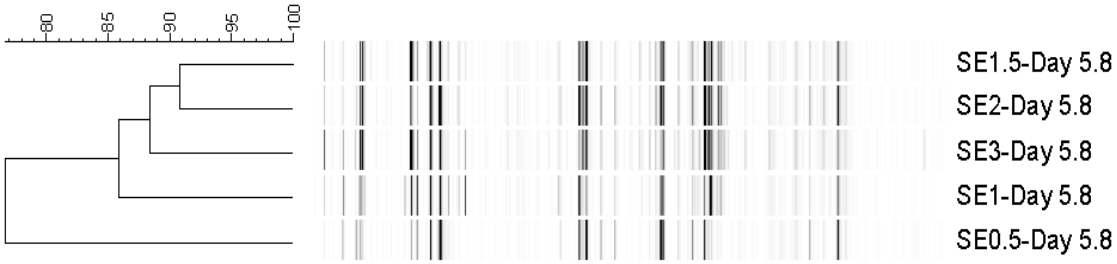


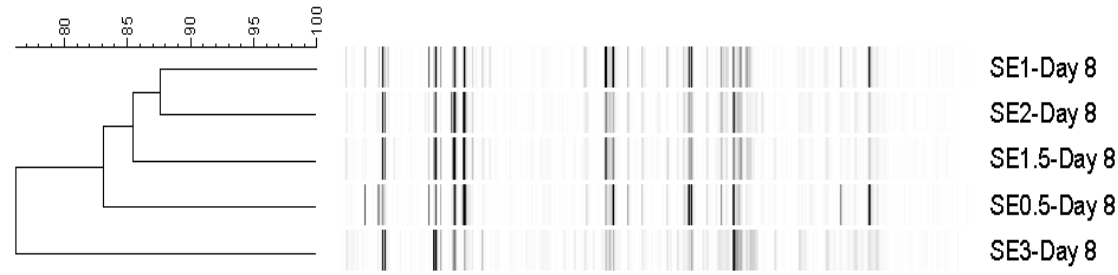
Figure 5.12 (Continued) A GelCompar II-generated UPGMA clustering dendrogram and corresponding normalized restriction profiles from the community DNA extracted from all the monitoring wells in the bioaugmented zone.

I. Day 5.8



J. Day 7

K. Day 8



L. Day 10

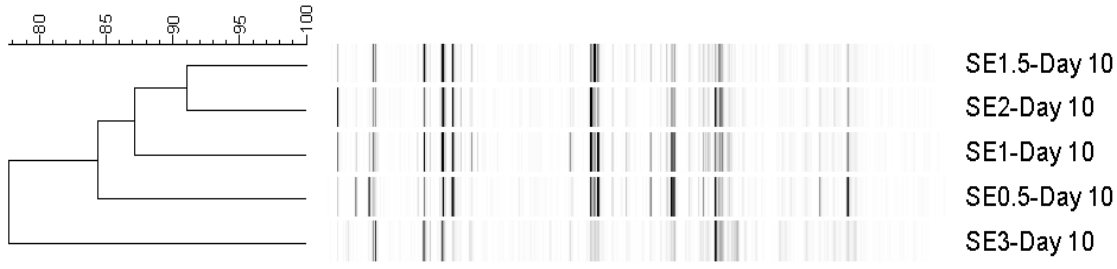


Figure 5.12 (Continued) A GelCompar II-generated UPGMA clustering dendrogram and corresponding normalized restriction profiles from the community DNA extracted from all the monitoring wells in the bioaugmented zone.

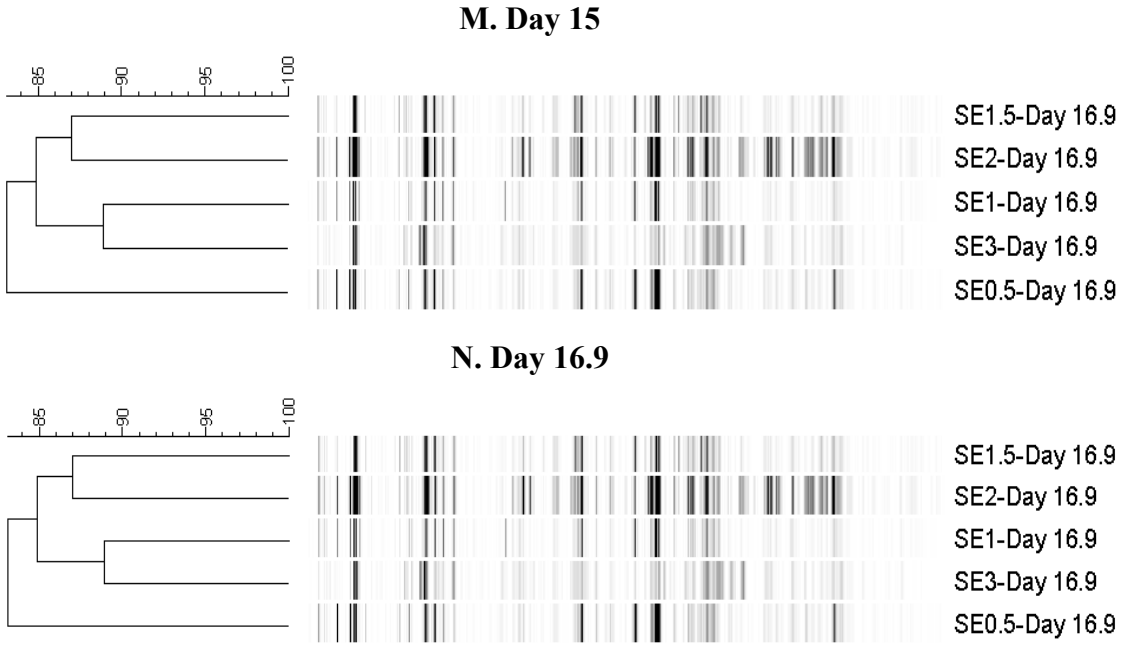


Figure 5.12 (Continued) A GelCompar II-generated UPGMA clustering dendrogram and corresponding normalized restriction profiles from the community DNA extracted from all the monitoring wells in the bioaugmented zone.

5.4 Discussion

Although the test conditions and purposes for the present bioaugmentation study were only slightly different from the October test, the December test exhibited several characteristics which were not shown previously. The October test showed that, at well SE1, butane was depleted within three days; whereas, during the December test, residual butane was still present through day 10 when the primary substrate was switched to propane. The slower butane consumption may have been due to competition between the primary and secondary substrate for the monooxygenase enzyme. In a previous study using microcosms bioaugmented with strain 183BP (Mathias, 2002), the reported similar

maximum transformation rates for butane and 1,1-DCE by strain 183BP at 2.5 μmol/mg/hr and 2.8 μmol/mg/hr respectively, which suggests the potential for where the other CAHs exhibited the lower maximum specific transformation rates of 0.49 μmol/mg/hr for 1,1-DCA and 0.20 μmol/mg/hr for 1,1,1-TCA.

Significant 1,1-DCE degradation was observed during the first few days without any apparent lag period, unlike the October test where a lag period of a few days was required for maximum TCA and DCA transformation. In a previous study of microcosms bioaugmented with strain 183BP (Mathias, 2002), 1,1-DCE was quickly transformed concurrently with butane, followed by 1,1-DCA and 1,1,1-TCA transformation once the butane was almost completely utilized. In a separate study using a butane-grown mixed culture, Kim et al. (2002) reported that the order of the half-saturation coefficients (K_s) from the highest to the lowest was 1,1-DCA, butane, 1,1,1-TCA and 1,1-DCE. The K_s of 1,1-DCE was about one order of magnitude lower than those for the other compounds. Therefore, 1,1-DCE may have exert greater competition for the monooxygenase enzyme than butane or the other CAHs. When studying CAH toxicity on methane utilizers, Henschler et al. (1979) also stated that the affinity of TCE for methane monooxygenase was higher than 1,1,1-TCA, which also was reflected by the higher degradation rate of TCE versus 1,1,1-TCA.

During the October test, maximum treatment efficiencies of 80% for 1,1,1-TCA and 96% for 1,1-DCA were achieved after butane was completely consumed as growth substrate in bioaugmented east leg. However, in this test, only 20% of the influent 1,1,1-TCA and 1,1-DCA, but 93% of the influent 1,1-DCE was transformed and complete butane utilization was not achieved by the first monitoring well. Although 1,1-DCE itself is not toxic, its transformation products are highly toxic (Dolan and McCarty, 1995). In a recent survey of aerobic cometabolism of CAHs by a butane-grown mixed culture, 1,1-DCE was rapidly transformed, but 1,1-DCE transformation caused greater cell inactivation than the transformation of other chlorinated ethenes (Kim et al, 2000). According to a

model developed by Anderson and McCarty (1996), transformation product toxicity appeared to be the most significant for 1,1-DCE among the CAH tested, including TCE and t-DCE. Although the 183BP cell concentrations in real-time PCR analyses was at least $(4.85 \pm 1.39) \times 10^3$ in well SE0.5, a gradual loss of CAH transformation ability was observed in 1,1,1-TCA and 1,1-DCA during the first ten days of the test. However, during the October bioaugmentation test, effective treatment of chlorinated ethanes was achieved under similar strain 183BP cell concentrations. The CAH transformation activity loss may have been due to the toxicity presented by accumulated transformation products from CAHs, especially from 1,1-DCE. A field evaluation of in situ CAH cometabolism by phenol-grown organisms conducted at the Moffett Field site in 1995 resulted in 50% transformation of the 65 μg/L influent 1,1-DCE and a 40% reduction in TCE removal efficiency due to DCE transformation (Hopkins and McCarty, 1995).

The primary substrate feeding was switched from butane to propane on day 10. A rapid decline of transformation activity for 1,1-DCE was observed in both well legs. Except for well SE2, strain 183BP cell concentrations in wells SE0.5, SE1 and SE1.5 increased from day 10 to day 15. This was possibly due to organisms had more affinity to butane as primary substrate and achieved better cometabolism capability. The increased cell concentration was inversely related to 1,1-DCE removal efficiency and may have been the result of detached cells from solid phases for two reasons: 1), accumulated toxicity of transformation products of CAHs; and/or 2), deficient physiological state caused by the substrate replacement. A microcosm study using aquifer solids and groundwater from the Moffett Field site and bioaugmented with an enrichment culture developed from Hanford DOE site, WA showed similar results (Rungkamol, 2001). It was found that the maximum transformation yields in microcosms bioaugmented with propane- and butane-utilizers were 0.019 and 0.036 mg 1,1,1-TCA/mg substrate, respectively.

With similar influent oxygen concentrations of approximately 40 mg/L, oxygen utilization was about 10 mg/L between the injection well and the first

monitoring well in this test. This was only about 50% of the utilization rate found in the October test, likely due to inhibition of butane oxidization by 1,1-DCE.

The cell density profile from the real-time PCR analysis revealed the spatial distribution of the 183BP cells in the monitoring wells. It was observed that increasing distances from the injection well corresponded to declines in the 183BP cell populations. Decreased butane concentration with distance limited the cell growth. High bromide recovery was observed in the all the monitoring wells (≥ 85%), thus confirming that dilution of the cells was insignificant. However, it is noteworthy that the cell density in the well SE2 was higher than that of well SE1.5 for the duration of the experiment. The reason for this contradiction might be that well SE1.5 had incomplete capture of the groundwater flow path. Furthermore, higher accumulation of toxic products from CAH transformation may have occurred with increased distance from the injection well, which could be a significant stress factor for cell growth.

The T-RFLP profiles showed the community structure transition over the duration of the bioaugmentation test. Although organisms with T-RFs of 47- and 254-bp were observed in community profiles and in the bioaugmentation culture, there was never a dominance or even strong representation of the augmented culture, indicating that they may not have adapted to the field environment and may not have significantly contributed to CAH degradation. Similar to the results from the October bioaugmentation test, the bacterial community in well SE0.5 shifted to two alternately dominating fragments, 277- and 126-bp. Apparently, the 277-bp fragment was associated with the fast degradation phase, while the 126-bp fragment dominated during the period of diminishing CAH transformation. Another difference is that 277-bp could be detected in the background groundwater samples in both tests and in samples from the west well leg, whereas the 126-bp was not. Similar trends were also observed in the other monitoring wells in the bioaugmented east zone with extended dominance time for the 277-bp with increasing distance from the injection well. The community shifts

were also evidenced by the cluster analysis. The 277-bp dominance was most likely due to favorable conditions established by the butane and oxygen additions; however, the 277-bp organism(s) may have been negatively affected by transformation products and diminished as the CAH transformation process proceeded. It is unknown why there was a distinct transition to dominance of the 126-bp fragment during the later phase of the experiment. CAH transformation efficiency decreased over the period of 126-bp dominance suggesting that the 126-bp organism(s) was unable to transform the CAHs or was inefficient at the process.

In a previous study conducted at the same site (Fries et al, 1997b), groundwater samples from three successive treatments (phenol + TCE + 1,1-DCE, phenol + TCE, and toluene + TCE) analyzed using amplified ribosomal DNA restriction analysis (ARDRA) with *Hae*III, *Hpa*II, and *Sau*3A as restriction endonucleases showed decreased species diversity when 1,1-DCE was included in the influent stream. The original diversity was re-established after TCE + toluene conditions were re-instituted in the absence of 1,1-DCE. The similarity of the banding patterns indicated that the community structure remained fairly stable when the site was treated with phenol or toluene. The predominant microbial taxa were found to be the beta proteobacteria *Variovorax*, *Azoarcus*, and *Burkholderia* and three gram positive groups including *Bacillus*, *Nocardia*, and an unidentified group.

In the present study, when butane was fed as primary substrate, the community T-RFLP pattern of the groundwater samples shared approximately 80% similarity. As the substrate was switched to propane on day 10, the bacterial communities in the groundwater samples collected thereafter showed about 75% similarity with butane-fed communities, indicating relatively stable community structure under two different substrates. However, in a study of soil community structure during bioaugmentation treatment using PCR-single-strand-conformation polymorphism analysis, drastic changes was observed between samples collected in the first two weeks (Cho and Kim, 2000). The community in the sample collected

in the first week of bioaugmentation treatment showed only about 50% identity to that of the second week, which was significantly lower than our observation at Moffett Field groundwater samples. This discrepancy may be due to the different habitats of the microbial communities and the experimental conditions at the two sites. No previous bioremediation studies in Moffett Field have reported the alternate dominance in microbial communities during a bioremediation test or similar phenomena.

CHAPTER 6
CONTINUOUS-FLOW SOIL COLUMN

6.1 Introduction

The objective of this research was to develop a quantitative SYBR Green I real-time PCR assay based upon 16S genes to provide assessment of the abundance and role of an augmented culture (stain 183BP) in 1,1,1-TCA biodegradation tests conducted at a continuous soil column and to characterize the bacterial community structure and possible community shifts corresponding to 1,1,1-TCA biodegradation using the Terminal Restriction Fragment Length Polymorphism (T-RFLP) method and statistical analysis.

6.2 Materials and methods

6.2.1 Soil column setup and sample measurement

. The soil column design and setup was described by Maremanda (2004). Briefly, the column consisted of a glass tube (25-mm inner diameter; 300-mm length) filled with aquifer solids taken from the Moffett Federal Airfield In-Situ Bioremediation Test Site, Mountain View, CA. Circulation of uncontaminated groundwater collected from the same site was carried out by a piston pump with a flow rate of 0.2 ml/min through the column. The groundwater was first saturated with oxygen at a level around 40 mg/L and stored in a metallized-film gas-sampling bag which was used as a reservoir. Groundwater saturated with butane was prepared in the same way except the groundwater was first sparged with nitrogen for 30 minutes to remove any aqueous oxygen. Chlorinated aliphatics (1,1,1-TCA and 1,1-DCE) were added to the reservoir to achieve concentrations of ~200μg/L or ~400 μg/L of 1,1,1-TCA and ~130 μg/L of 1,1-DCE.

The concentrations of butane, DO and chlorinated aliphatics were measured in the influent and effluent samples. An approximate volume of 300 ml effluent was collected twice each week to perform microbial analyses. Gaseous samples were analyzed using a Hewlett Packard (Wilmington, DE) 6890 gas chromatograph (butane, flame ionization detector [FID]; 1,1,1-TCA and 1,1-DCE, ^{63}Ni electron capture detector [ECD]). A microelectrode DO probe (model #: 16-1730; Microelectrodes Inc., NH) and a DO meter were used to measure the DO concentrations at the effluent end of the column.

6.2.2 Microbial analysis

The methods for microbial analysis were detailed in Chapter 4.2. Briefly, the community 16S rDNA was extracted from effluent samples used for real-time PCR and T-RFLP analyses. The 183BP-sepefic primers, Ran191F and Ran44R, were used for real-time PCR reactions; whereas, the universal primers, 27F-B-FAM and 338Rpl, were used for T-RFLP analysis.

Initially, real-time PCR was performed on triplicates of each sample at three serial dilutions in the same plate, 1:1, 1:10 and 1:100. When PCR inhibition did not occur, the C_T values of 1:1 and 1:10 dilutions were very similar. However, the presence of humic or other background compounds in the environmental samples often inhibited the PCR reaction in the 1:1 dilutions. A 10-fold decrease of 183BP cell concentrations was observed between 1:10 and 1:100 dilutions for most samples. Therefore, these two dilution ratios were for cell quantification.

6.3 Results

6.3.1 CAH degradation and strain 183BP quantitation

Concentrations of 1,1,1-TCA, DO, butane, and strain 183BP cells were monitored at the effluent point on a routine basis, and were plotted as a function of

time (Figure 6.1, Figure 6.2 and Figure 6.3). The column was continuously fed 1,1,1-TCA at about 200 µg/L except from day 30 to day 53 when the influent concentration was approximately doubled. Transformation studies were performed in three scenarios: Phase I from day 0 to day 30, Phase II from day 30 to day 65, and Phase III from day 65 to 115. Additionally, from day 120 to day 135, the column performance at 1,1,1-TCA transformation was evaluated with the presence of 1,1-DCE at a influent concentration of approximately 140 µg/L.

Prior to bioaugmentation, hydraulic characterization of the column was conducted using bromide, oxygen, butane and 1,1,1-TCA. It took 3.6 hours for bromide to reach 50% breakthrough and a porosity of 0.26 and a dispersion coefficient of $1.96 \times 10^{-3} cm^2/s$ were obtained from a 1-D transport model analysis of bromide transport tests. It took 11 days for DO to reach a maximum concentration of approximately 63% of the influent value. 1,1,1-TCA concentrations approached the influent value within about 4 days. The transport of 1,1,1-TCA was observed to be retarded, with a retardation coefficient of 3.1, and no evidence of 1,1,1-TCA transformation was observed prior to bioaugmentation and biostimulation of the column. One day prior to bioaugmentation, the column was fed with cyclical 2-hr oxygen pulses followed by 0.5-hr butane pulses. The butane mass balances indicated that no butane was consumed. Bioaugmentation was initialized on day 8 with strain 183BP as inoculum. About 2.5 ml of culture was added in increments of 0.5 ml over a span of 2.0 hours. The total mass of the cells added was 0.9 mg. Unfortunately, due to failure in FISH analysis, no microbial data were obtained for the first 35 days of the test. From day 37 on methods based upon bacterial 16S rDNA including real-time PCR and T-RFLP were used to perform 183BP cell quantitation and to investigate the bacterial community kinetics.

Upon bioaugmentation, butane began to decrease along with DO. DO decreased to approximately 20% saturation on day 11, and butane decreased to below detection limits. At least 85 % removal of the 200 µg/L influent

1,1,1-TCA was observed 10 days after bioaugmentation and was maintained over a 15-day period.

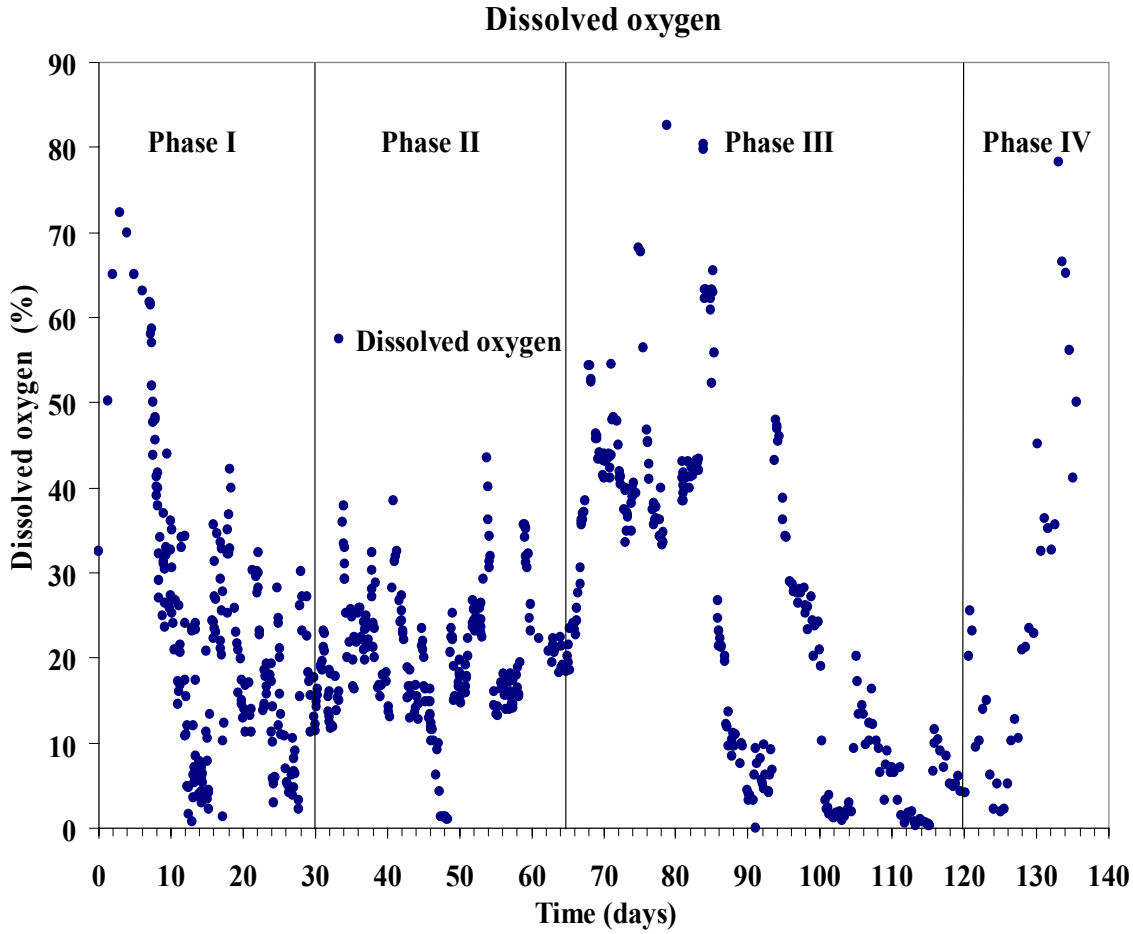


Figure 6.1 Profiles of dissolved oxygen in the soil column. Influent dissolved oxygen was 75.5% saturation (~40mg/L). (data were from Maremanda, 2004)

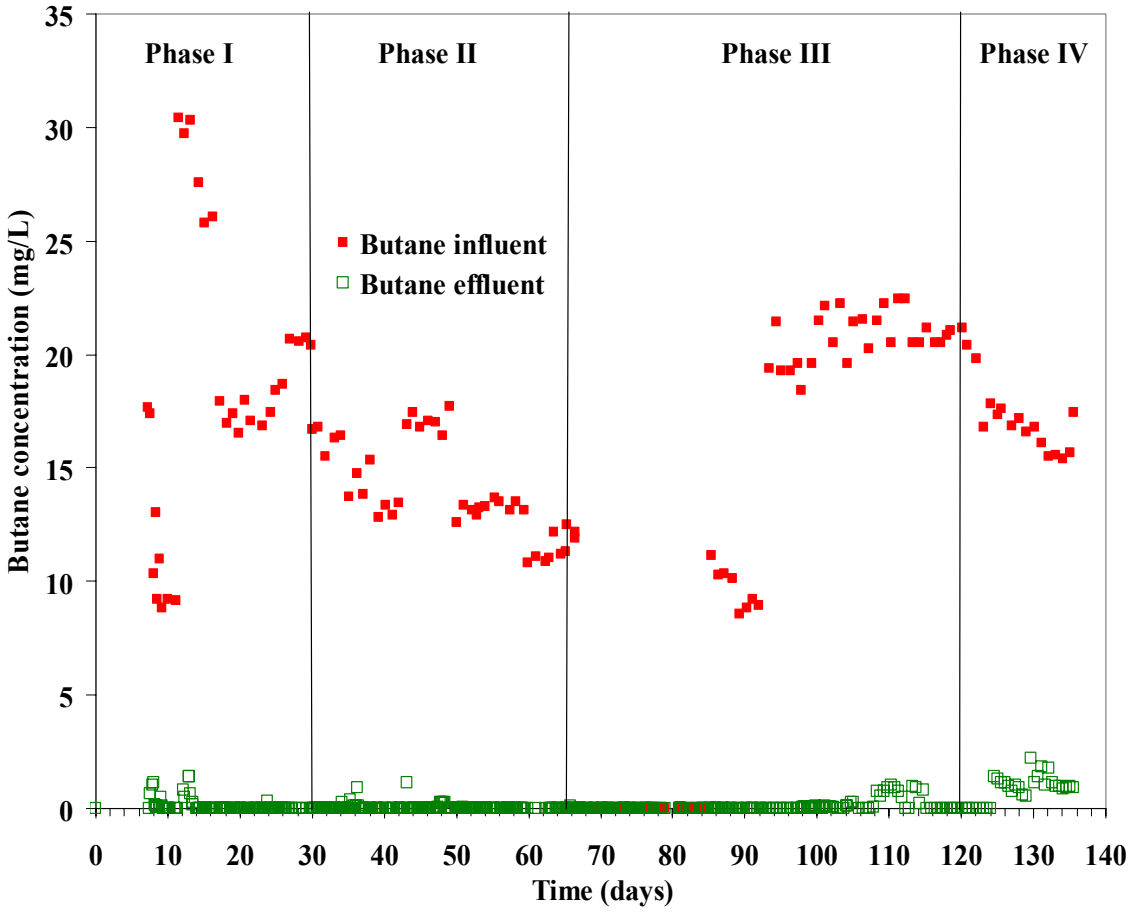


Figure 6.2 Profiles of butane concentrations in the soil column (data were from Maremanda, 2004)

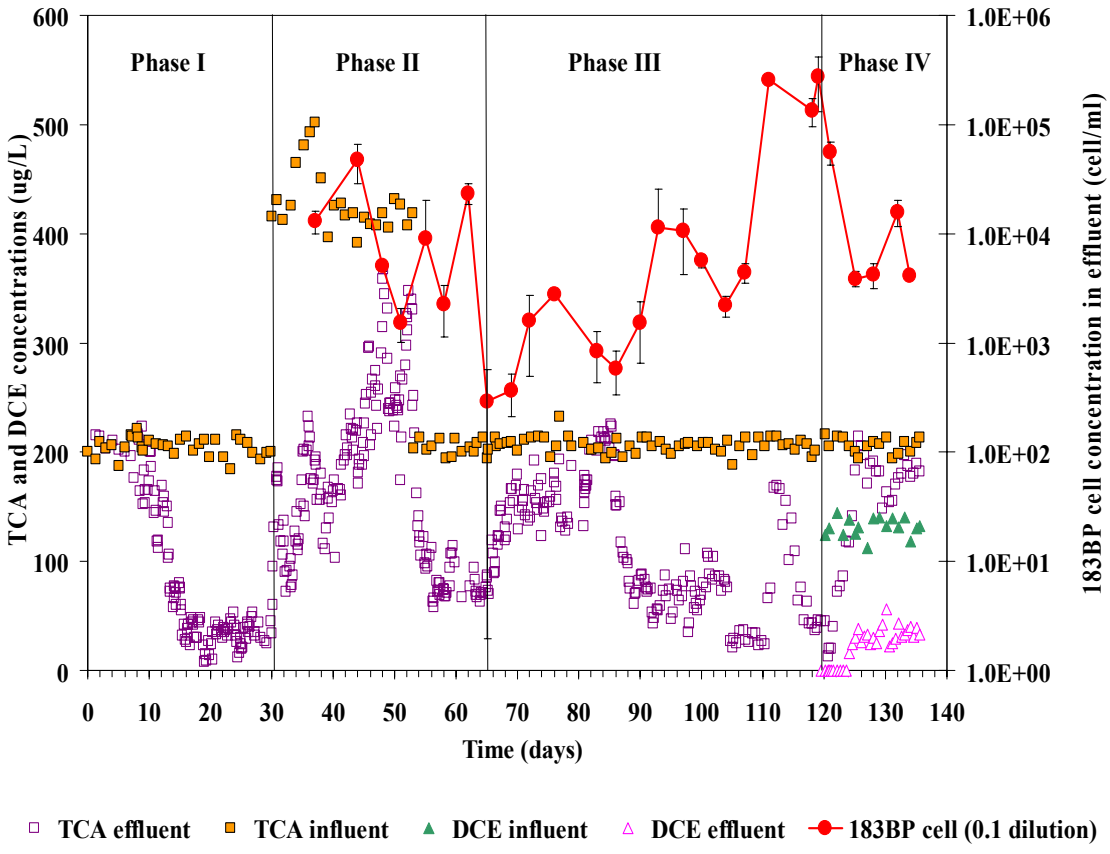


Figure 6.3 Profiles of 1,1,1-TCA, 1,1-DCE and the strain 183BP cell concentrations in the soil column (1,1,1-TCA and 1,1-DCE data were from Maremanda, 2004)

From day 30 to day 53, the influent 1,1,1-TCA concentration was raised to 400 μg/L. With the influent concentrations ranging from 11 mg/L to 17 mg/L, butane was gradually consumed in the soil column. Trace butane concentrations (≤ 0.8 mg/L) were observed around day 35 which may have been due to stressed butane utilizing population under high 1,1,1-TCA toxicity. However, high variation of effluent DO concentration was also observed. The column began to lose transformation ability evidenced by the increasing 1,1,1-TCA concentrations in the effluent. Time to 50% 1,1,1-TCA breakthrough was about 12 days after the increase in influent concentration. By day 53, only about 13% of the influent 1,1,1-TCA was effectively removed. No direct information of changes in the cell density during this period could be concluded because of the limited number of microbial samples.

In order to investigate if the column could regain its transformation ability, the influent 1,1,1-TCA concentration was reduced back to 200 μg/L on day 53 and maintained at this level through day 65. Effluent 1,1,1-TCA concentration dropped rapidly and a near-steady-state concentration of about 80 μg/L was achieved from day 56 to day 65, resulted in 60% 1,1,1-TCA removal in the column. During this period, real-time PCR results showed that the 183BP cell concentration estimated from 1:10 dilution rose up from approximately 9×10^3 cells/ml on day 55 to 2×10^4 cells/ml on day 62. However, a clear two-log-order decline of the cell concentration to approximately 300 cells/ml occurred on day 65. This might be caused by low influent butane concentration. Very good agreement was achieved between 1:10 and 1:100 dilutions in estimation of cell numbers.

1,1,1-TCA transformation stability was also evaluated without electron donor present. No butane was fed from day 66 to day 85. The butane shut-down subsequently resulted in reduction in oxygen utilization and the effluent DO concentration approached the influent value of 80% saturation by day 83. Without the presence of a primary growth substrate, 1,1,1-TCA treatment declined and the effluent concentration reached the influent value of 210 μg/L on day 85, though a

residual removal efficiency of 22% was observed during much of this period. The effluent concentration of 183BP cells rose during this period from about 270 cells/ml on day 69 to 3000 cells/ml on day 76 possibly as a result of primary substrate limitation causing sloughing of bacterial cells from the aquifer solids.

On day 85 the 0.5-hr butane pulse followed by 2-hr DO pulse was restored as in phase I and II, to test whether the bioaugmented culture could regain its activity and transform 1,1,1-TCA. Approximately 70% of the influent 1,1,1-TCA was removed by day 93. A 2-log-order increase in the cell density from $(5.85 \pm 2.50)\times10^2$ cells/ml on day 86 to $(1.13 \pm 1.43)\times10^4$ cells/ml on day 93 was observed, concomitant with the decline of effluent 1,1,1-TCA levels.

Neither the 1,1,1-TCA removal efficiency nor the cell concentration changed significantly when influent butane concentration was doubled from day 93 to 97. However, after butane pulses increased to 40 min from day 97 to day 114, about 85% of the influent 1,1,1-TCA was treated during a 5-day period from day 105 to day 110, followed by a gradual loss of transformation ability. During this period, a considerable amount of DO was utilized and the column was almost driven anaerobic by day 110. A sharp transient increase in the cell density occurred on day 111 to a level of about 3×10^5 cells/ml, the maximum concentration observed throughout the test. The escalating cell density may have resulted from that oxygen limitations causing large scale sloughing of bacterial cells from the solid phase. About 1mg/L butane and no dissolved oxygen was detected in the effluent flows from day 109 to day 114, and the increased effluent cell density did not correlated to increased 1,1,1-TCA removal capacity, but rather the reverse. Similar post-PCR DNA banding results were obtained from a traditional PCR assay (Figure 6.4).

On day 114 the butane pulse was returned to 20 min with an influent concentration of around 20 mg/L plus a 2-hr pulse of oxygen for the next 6 days. The column regained 1,1,1-TCA transformation ability and about 79% removal efficiency

was achieved on day 118. Effluent DO concentration continued to remain low (~10% saturation), but complete butane consumption was observed. The average 183BP concentration was approximately 1×10^5 cells/ml with some fluctuation.

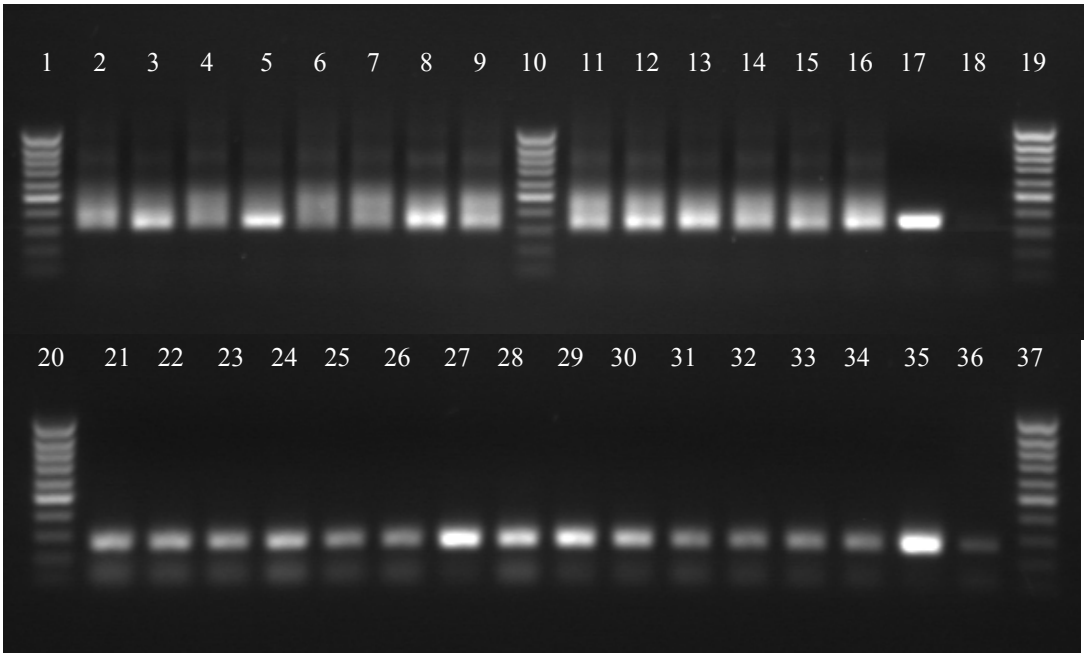


Figure 6.4 Detection of strain 183BP populations in the soil column effluent with universal bacterial primers and 183BP-specific primers. Lane 1, 10, 19, 20 and 37, 80- to 1032-bp DNA mass ruler ladder (Fermentas); lane 2 to 9 and 11 to 16, effluent samples amplified by the universal bacterial primers (day 90 to day 134); lane 21 to 34, effluent samples amplified by the 183BP-specific primers (day 90 to day 134); lane17 and 35, positive controls; lane 18 and 36, negative controls.

During Phase IV, 1,1-DCE was injected at an influent concentration of 130 µg/L with 1,1,1-TCA at about 200 µg/L beginning on day 120. Complete butane utilization was observed for the first 5 days, followed by effluent butane levels rising to approximately 1 mg/L throughout the remainder of the test. The oxygen consumption rate declined to only 10% utilization of the influent DO concentration by the end of the test. Significant 1,1-DCE transformation but little 1,1,1-TCA removal was observed. Approximately 80% effective treatment of influent 1,1-DCE was observed during the 14-day operation. Similar to the field observations, effluent 1,1,1-TCA concentrations gradually increased for the first 6 days and remained close to the influent level for the rest of the test. Time to 50% 1,1,1-TCA breakthrough was about 3.3 days. During this period, the 183BP concentration stayed a level of approximately 5.5×10^3 cells/ml.

6.3.2 Community T-RFLP profiles analysis

T-RFLP analysis was conducted on all of the soil column effluent samples using universal bacterial primers (27F-B-FAM and 338Rpl) and *Mnl*I and *Hin6*I restriction enzymes. However, analyses were primarily based upon the *Mnl*I T-RFLP profiles since they provided the highest discrimination of fragments due to increased frequency of recognition sites and the short length of the amplicons used.

Similar to the field groundwater samples, no fragments of 183 bp length were observed in any of the effluent samples throughout the test. However, unlike the

field test, T-RFLP profiles of the bacterial community in the soil column exhibited greater diversity without clear species dominance or succession. The 126-bp peak, one of the major T-RFLP fragments found in field groundwater samples, was present only in trace amounts. Only one sample obtained on day 76 showed a 277-bp-dominance T-RFLP profile.

From day 37 to day 51 when the influent 1,1,1-TCA concentration was about 400 μg/L and only 13% was removed , it was shown that 47- and 253-bp fragments were the largest peaks and accounted for about 9% and 12% of the total area of all the detected peaks, respectively (Figure 6.5). Although the 47-bp fragment never obtained dominance, it accounted for a fairly stable percentage of total peak areas throughout the test period. The area percentage of the 167-bp peak was about 10% for day 37 and day 44 but diminished by day 51. Similar patterns were observed for the 278-bp fragment.

On day 53, 1,1,1-TCA concentration was dropped down to 200 μg/L and maintained there through day 65. The utilization rates for the influent butane and DO were about 100% and 74%, respectively. During this period, the 253-bp fragment became the largest peak on day 62 and day 65m and increased from about 7% of the total peak area on day 55 to 30% on day 65. The 47-bp peak also increased from day 53 to day 65, but encompassed a smaller percentage of the total population than it did. On day 58, upon recovery of TCA treatment, a 165-bp fragment was found during day 37 to day 55 to be the largest peak in the profile, at 15.6% of total peak area.

The T-RFLP data showed a more diverse community structure when butane delivery was interrupted between day 66 and day 85. For instance, 130 peaks were detected in the sample collected on day 69; whereas, the average number of detected peaks in all effluent samples was 51. On day 76 in the absence of butane and little TCA removal, the 277-bp peak was dominant and accounted for about 22% of the total peak area. Upon restart of butane addition on day 85, about 75% reduction in the species diversity was observed immediately. Three peaks at 237-, 279- and 283-bp dominated the bacterial community on day 86 and accounted for 23.2%, 20.4% and 16.4% of the total peak area, respectively. On day 93 and 97, 44.6% and ~20%, respectively, of the total peak area was attributed to a 274-bp fragment, that, although present in most samples, had not gained prominence previously. Two unique features of the T-RFLP pattern were observed from the

sample obtained on day 100: 1) The least diversity of any sample with only 16 different fragments; and, 2) the 279-bp fragment accounted for 64.9% of the total peak area, the largest percentage found during the test. Beginning on day 120, 1,1-DCE was delivered to the column simultaneously with 1,1,1-TCA. After about 10 days, TCA removal had dropped significantly, but DCE transformation continued and the microbial community was dominated by fragments of 237-, 253-, and 279-bp.

T-RFLP banding patterns were also analyzed using GelCompar II (Applied Maths, Belgium). It was shown that all the samples showed at least 84% similarity in T-RFLP patterns (Figure 6.6). Unlike the microbial community succession observed in the field groundwater samples, clusters generated from the T-RFLP patterns of the column effluent samples did not clearly reflect the operational conditions or CAH degradation progress. Samples were also clustered according to the bioaugmentation treatment phases (Fig 6.7). For instance, during the second operational phase, the samples collected during this period were grouped together on a level of approximately 90% similarity. To investigate the resemblance of the bacterial community under similar chemical conditions, the samples were also analyzed according to the effluent 1,1,1-TCA and DO concentrations (Figure 6.8). Under low oxygen levels (≤ 10% saturation), samples had at least 89% similarity in T-RFLP patterns. There was at least 84% similarity in the T-RFLP patterns of the samples collected when low 1,1,1-TCA concentrations were detected in the effluent.

6.4 Discussion

During the whole experiment, 1,1,1-TCA treatment efficiency was evaluated under various conditions, including changes of dissolved oxygen and butane levels, and presence of 1,1-DCE. At least 85 % 1,1,1-TCA reduction in the injection concentrations of 200 μg/L was observed 10 days after bioaugmentation and maintained steadily over a 15-day period. During this period, the time-averaged

butane injected into the column was about 3 mg/L. Unlike a typical 3- to 4-day lag time of 1,1,1-TCA transformation in the field observations, upon bioaugmentation effluent 1,1,1-TCA concentration immediately declined in the column. However, the time-averaged butane concentration in the field groundwater samples was about 2 to 4 mg/L before complete depletion. Similar TCA treatment efficiencies were observed in the field, but were not maintained over time as they were in the soil column. Unfortunately, no microbial data was available during this operational phase.

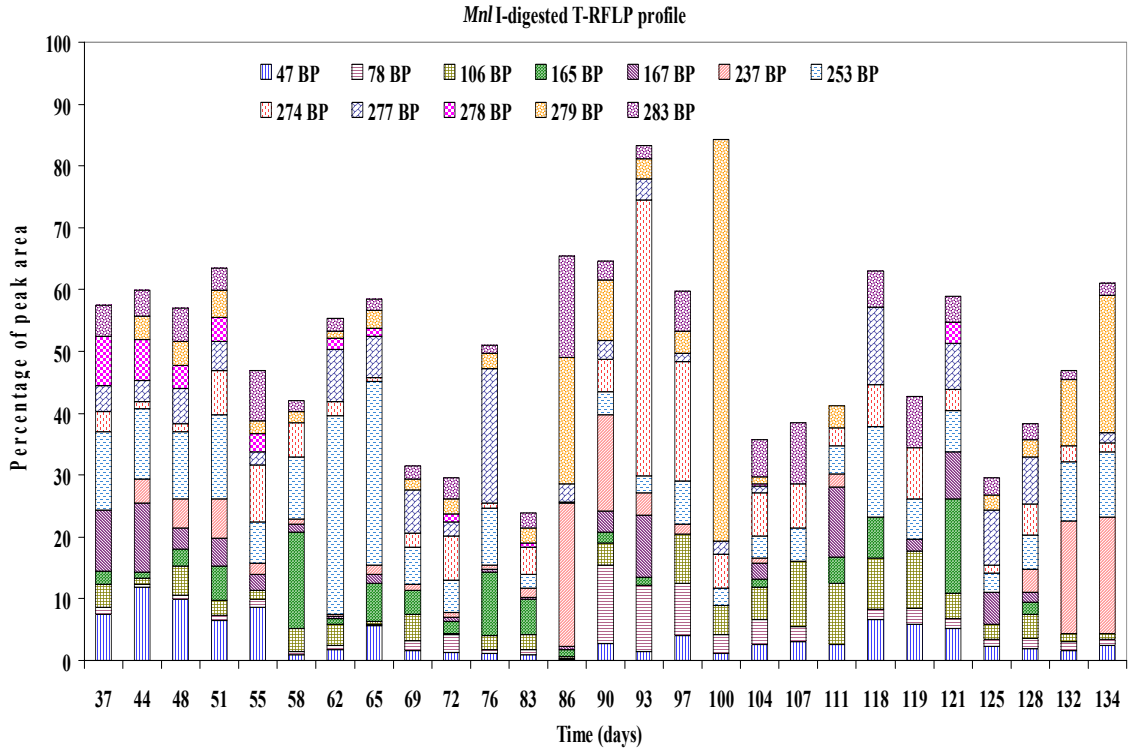


Figure 6.5 Changes in the bacterial community structure during the bioaugmentation test in the soil column (from day 37 to day 134).

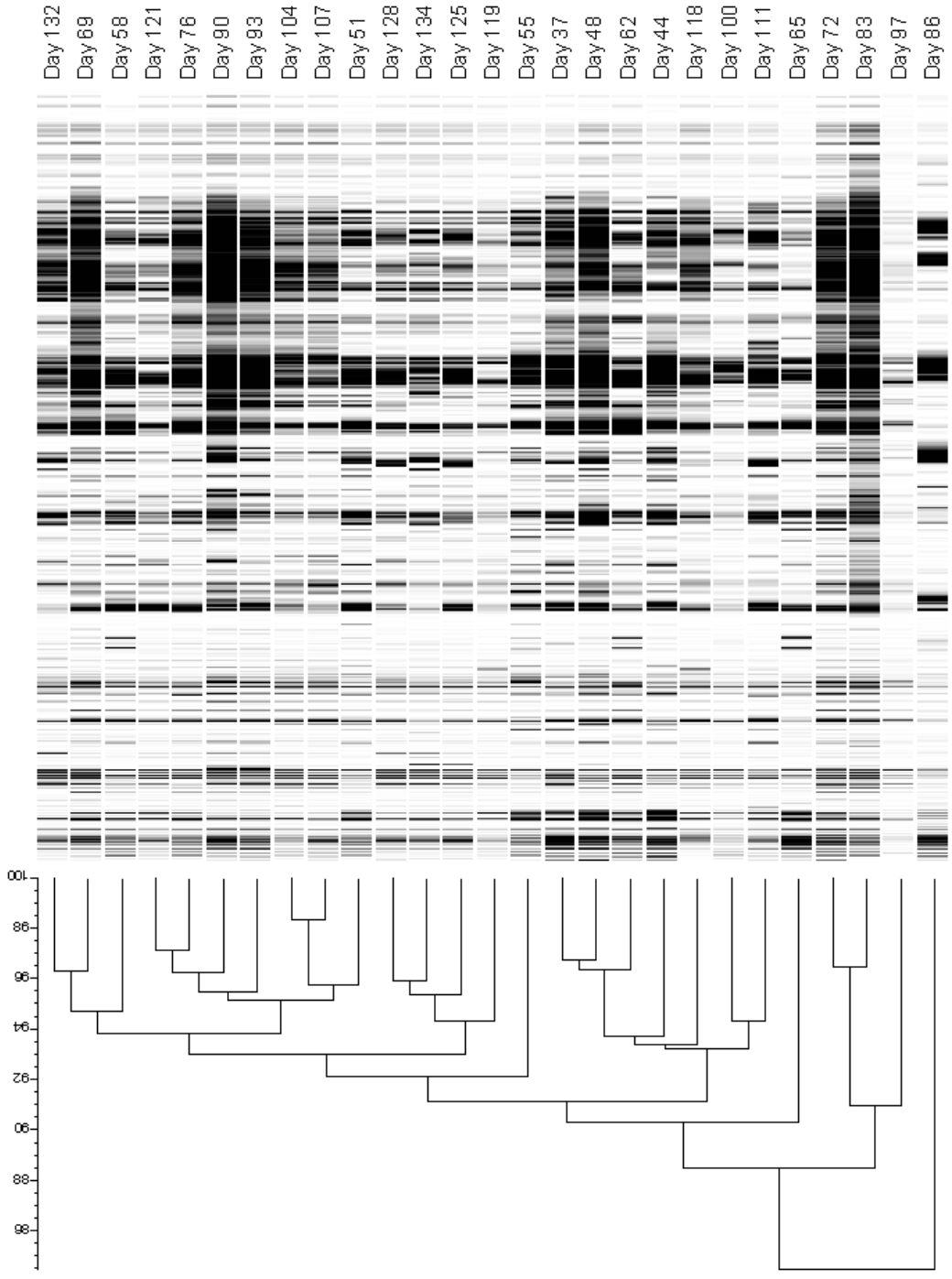


Figure 6.6 A GelCompar II-generated UPGMA clustering dendrogram and corresponding normalized restriction profiles from the community DNA extracted from the soil column effluent.

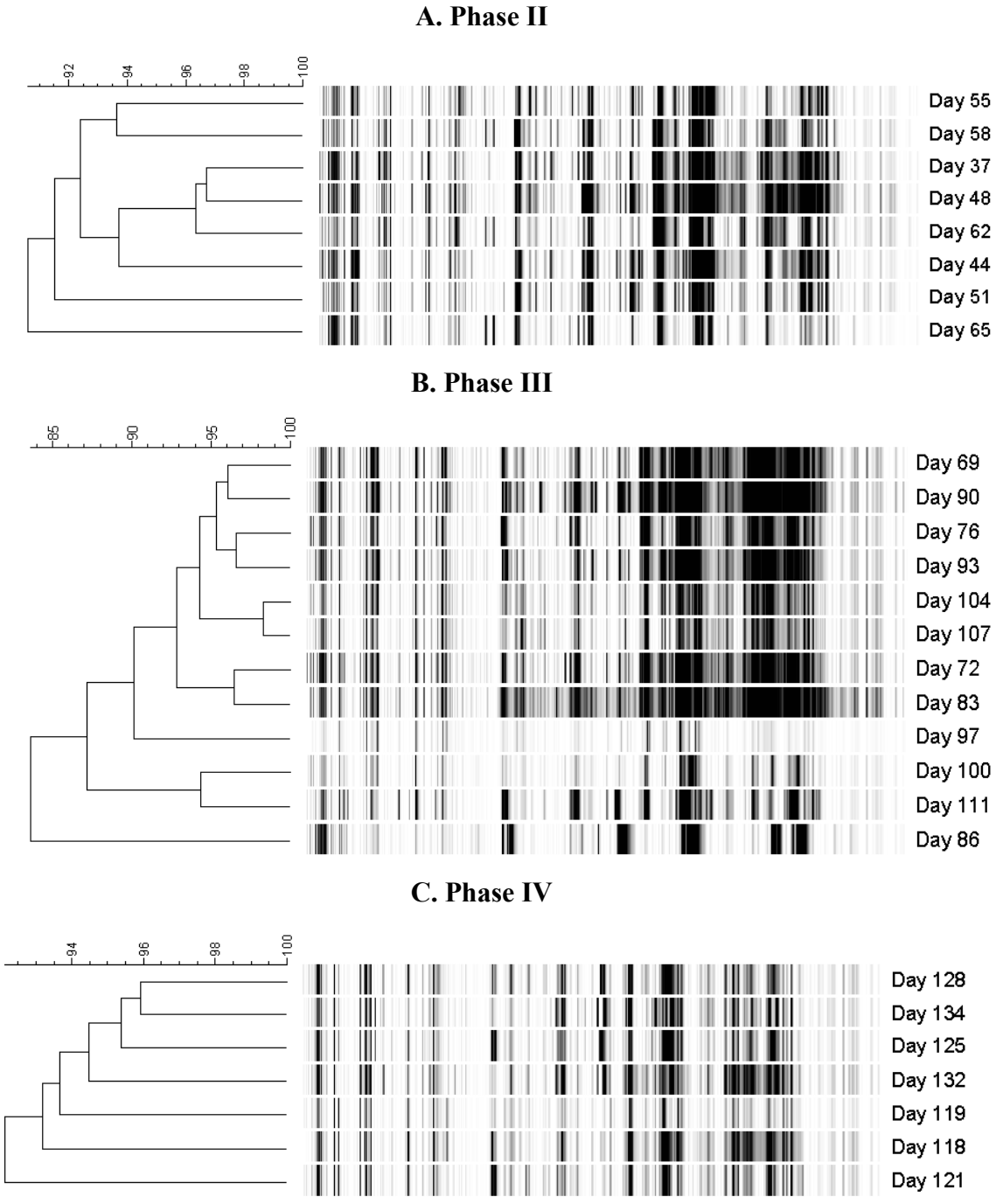
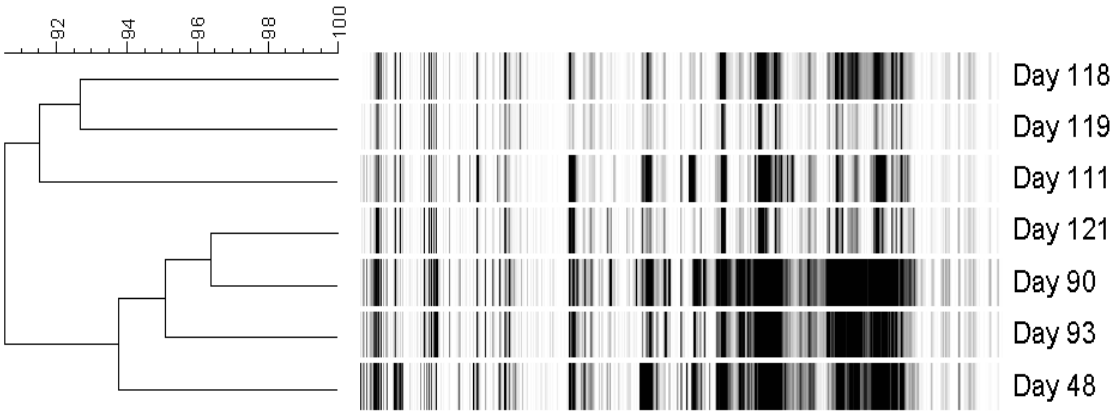


Figure 6.7 A GelCompar II-generated UPGMA clustering dendrogram and corresponding normalized restriction profiles from the community DNA extracted from the soil column effluent and assorted in three bioaugmentation treatment phases (Phase II, III, and IV).

A. Oxygen concentration ≤10%



B. 1,1,1-TCA ≤ 65µg/L in the effluent

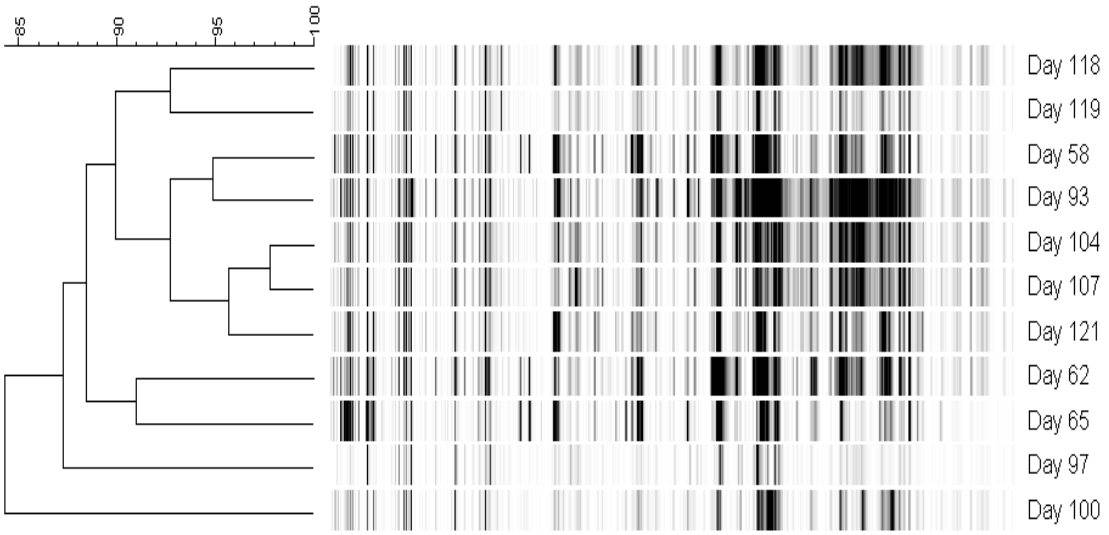


Figure 6.8 A GelCompar II-generated UPGMA clustering dendrogram and corresponding normalized restriction profiles from the community DNA extracted from the soil column effluent and assorted in similar environmental conditions (effects of oxygen depletion and 1,1,1-TCA transformation ability).

When 1,1,1-TCA concentrations were increased to 400 μg/L, TCA transformation efficiency decreased over time. Broholm et al (1990) studied toxicity effects of 1,1,1-TCA and trichloroethene (TCE) on a mixed methane-utilizing culture, and found that the increased concentration of each CAH caused decreased consumption of methane, resulting from a competition between methane and the secondary substrate for the same enzyme, methane monooxygenase. In previous microcosm and modeling studies (Mathias, 2002), the estimated 1,1,1-TCA transformation capacity for the bioaugmentation culture was 0.11 mg TCA/mg cells and, when combined with a microbial yield, become the transformation yield of 0.1 mg TCA/mg butane. The calculated transformation yield in the column was similar to the number estimated from the microcosm studies. On a total mass basis, more TCA was transformed at higher influent concentrations than lower concentrations, but high influent TCA concentration caused an overall decrease in removal efficiency.

Unfortunately, no direct information can be concluded from the limited microbial data during this period. But the strain 183BP cell concentration from day 37 to day 51 was at least 1.5×10^3 cells/ml. The October field bioaugmentation test showed that effective treatment of 1,1,1-TCA (~75% removal of a influent concentration 200 μg/L) was achieved under similar strain 183BP cell concentrations. A accumulation of toxic CAH transformation products may have damaged the cells' cometabolism ability or their overall viability. Upon decreasing the influent concentration to 200 μg/L, effluent TCA concentrations reached steady-state levels of 80 μg/L representing about 60% removal. The loss of transformation efficiency may be related to toxic effects and decreased cell concentrations during the high 1,1,1-TCA feeding or to competitive effects by stimulated indigenous butane-utilizing, non-TCA-transforming populations. Effects from the high concentration of 1,1,1-TCA in the soil column remained for an extended period, although the influent concentration was reduced to previous values.

1,1,1-TCA transformation was shown to be linked to butane addition. When butane addition was shut down, the 1,1,1-TCA removal efficiency declined immediately. Since the culture used butane as primary substrate and transformed CAHs as secondary substrates, the removal of butane stressed the cells. During this period, the strain 183BP cells concentration in the effluent increased to a level of 3.5×10^5 cells/ml on day 62. The increased cell concentrations were inversely correlated to the decline in the 1,1,1-TCA removal rate. The higher cell numbers may have been attributed to cell inactivation or starvation and subsequent sloughing off of the aquifer solids. Since neither the DNA extraction protocol nor the PCR reaction can distinguish the physiological state of the cells, the high cell numbers may have included inactive or dead cells. Effects of butane removal can be seen from T-RFLP analysis, too. The number of the bacterial species detected by the T-RFLP methods increased by at least 100% over the average 51 peaks per sample in the sample set. Presumably, this was the result of switching from a concentrated source of a single organic substrate to multiple low-concentration sources of complex organics from cell lysis and decay.

Once the butane delivery was re-started, about 70% treatment of 200 μg/L influent 1,1,1-TCA was regained, indicating that the loss of TCA transformation efficiency was reversible as long as butane was re-supplied. The strain 183BP cell density stopped declining and increased 1-log-order in magnitude to approximately 3000 cells/ml on day 76. Treatment efficiency was less than the maximum seen in Phase I of ~85% removal, but still reasonably good.

Oxygen was also found critical to the culture's performance on CAH transformation. Decreased DO concentrations caused the 1,1,1-TCA concentration to increase to influent values. Under such conditions, the culture was not able to completely consume the added butane, evidenced by the 1mg/L butane detected in effluent samples.

1,1-DCE, an abiotic transformation product of 1,1,1-TCA, can have adverse impact on 1,1,1-TCA biodegradation (Kim et al, 2000; Rungkamol,

2001;Mathais, 2002; Lim, 2003). The column data showed that the presence of 1,1-DCE caused inhibitory effects on 1,1,1-TCA biotransformation. As much as 85% of influent 1,1-DCE (~130 µg/L) was removed through 10 days duration of the experiment. However, 1,1,1-TCA concentrations in the effluent gradually approached the influent value and remained about 10% removal efficiency was achieved throughout the rest of the experiment. 1,1,1-TCA and 1,1-DCE transformation in the field bioaugmentation conducted in December, 2003, was in a close agreement to the laboratory column observations. During this period, the strain 183BP cell concentrations stayed at a near-steady level of approximately 3×10^3 cells/ml in the column effluent. These concentrations were in the range of the cell concentrations in field groundwater samples. The faster transformation of 1,1-DCE over 1,1,1-TCA may be due to the kinetic characteristics of the monooxygenase enzyme system. In a microcosm bioaugmented with strain 183BP cells and modeling study, Mathis (2002) reported the maximum utilization rates for 1,1,-DCE, butane and 1,1,1-TCA were 2.8, 2.5 and 0.2 µmol/mg/hr. The K_s value of 1,1-DCE (1.48 µmol/L) was also lower than that of 1,1,1-TCA (12.2 µmol/L). In a transformation study of 1,1,1-TCA, 1,1-DCA and 1,1-DCE by a butane-grown mixed culture (Kim et al, 2002), it was found that the butane culture had higher maximum transformation rate (k_{max}) and lower half-saturation coefficient (K_s) for 1,1-DCE than 1,1-DCA and 1,1,1-TCA. Other studies have also been conducted to evaluate the transformation kinetics of CAH mixtures. Aziz et al (1999) used *Methylosinus trichosporium* OB3b PP358, which constitutively expresses soluble methane monooxygenase (sMMO), to study the CAH degradation kinetics. They found that this bacterial strain degraded trichloroethylene (TCE), chloroform, cis-1,2-dichloroethylene (c-DCE), trans-1,2-dichloroethylene (t-DCE) and 1,1-DCE rapidly, with maximum substrate transformation rates of >20.8, 3.1, 9.5 24.8, and >7.5 mg/mg-day, respectively. However, 1,1,1-TCA was not significantly degraded.

The T-RFLP profiles of the bacterial community in the soil column indicated that, over the entire experiment, no significant community shifts were observed to include dominant presence of the inoculated strain 183BP. Although community composition shifted often, it was difficult to correlate community shifts to differences in operational conditions. In past, this was due to the frequency of changes constituted concentration (i.e., butane, DO, TCA), the time between microbial sampling events, and perhaps the travel time between the active region of the column and column effluent point.

One of the biggest challenges encountered in the use of quantitative real-time PCR was that the technique could not distinguish between functional or dysfunctional cells, and live or dead cells. Under some conditions, such as influent butane removal and oxygen depletion, the performance of strain 183BP on the 1,1,1-TCA biodegradation cannot be supported by the changes in the cell densities. Furthermore, the conversion of the fluorescent signals to the actual concentration of the quantified population is a possible source of measurement error. Since the thermocycler only allows a 96-well plate per run, the large number of the samples did not allow us to quantify all the samples solely on one calibration curve. Thus, the comparison of samples from different plates can be influenced by interassay variability (Giulieffi et al., 2001). Additionally, cell densities in the groundwater phase may not accurately represent cell populations attached to aquifer solids.

CHAPTER 7
CONCLUSION

Several molecular-based methods, including PCR, real-time PCR and T-RFLP, were used to identify and quantify strain 183BP populations under natural and laboratory conditions and to investigate bacterial community composition changes resulting from stimulation of a contaminated aquifer zone with butane and oxygen to enhance CAH cometabolism. Two primers, Ran191F and Ran443R, were designed to specifically target strain 183BP during PCR and to exclude other bacterial species. Although the specificity analysis indicated that the primer pair also matched sequences of six other known bacterial strains, melting curve analyses indicated that only one of many environmental and laboratory samples (<0.7%) generated identifiable non-target amplicons during real-time PCR analyses.

A sensitive assay based on real-time SYBR Green I PCR detection was successfully developed to provide quantitative information on strain 183BP populations in field groundwater samples and soil-column effluent samples. Standard curves showed a linear relationship between cell concentrations and cycle threshold values, C_T, during real-time PCR, resulting in a cell quantification detection limit of approximately 100 cells/ml and a presence/absence detection limit of 2 cells/ml. DNA extracted from samples obtained from the field and a soil column was diluted 1:10 prior to real-time PCR analysis to minimize PCR inhibition by unknown constituents present in the groundwater samples.

Bioaugmentation of a mixed butane-utilizing microbial culture to the subsurface at Moffett Federal Airfield In-Situ Bioremediation Test Site (Mountain View, CA) resulted in successful enhanced biotransformation of the chlorinated aliphatic compounds 1,1,1-TCA, 1,1-DCA, and 1,1-DCE. Maximum contaminant removal efficiencies were 80% for 1,1,1-TCA (200 µg/L influent concentration), 96% for 1,1-DCA (175 µg/L influent concentration), and 93% for 1,1-DCE (175

µg/L influent concentration). However, treatment efficiencies were not sustainable over extended periods of time. Maximum treatment generally occurred between 4 and 8 days after bioaugmentation and gradually declined with time. The time to loss of activity varied from about 3 weeks in the October 2003 test, that did not include 1,1-DCE, to about 2 weeks in the December 2003 test where 175 µg/L DCE was present. In contrast, operation of the non-bioaugmented well leg resulted in essentially no 1,1,1-TCA or 1,1-DCA transformation and only about 25% removal of 1,1-DCE.

The presence of 1,1-DCE significantly inhibited butane oxidation and cometabolic transformation of the other CAHs present. In the October test in the absence of 1,1-DCE, complete butane utilization and CAH transformation occurred by the first groundwater monitoring well 1 m from the injection well, with maximum 1,1,1-TCA and 1,1-DCA removal efficiencies of 80% and 96%, respectively. In the December test with influent 1,1-DCE concentrations of 175 mg/L, 1,1,1-TCA and 1,1-DCA maximum removal efficiencies dropped to about 60% and butane utilization and CAH transformation extended beyond the first monitoring well. Approximately 60% of the 1,1-DCE transformation occurred before the first monitoring well while another 30% removal occurred between the first and second monitoring wells.

In the October 2003 test during the effective treatment period, the strain 183BP cell concentration was above 900 cells/ml as measured in well SE0.5. The 183BP cell concentration declined with time, concomitant to the gradual loss of CAH transformation ability. No T-RFs of 183-bp length were detected in any of the groundwater samples during T-RFLP analyses and only transient appearance of small peaks were found for any of the bioaugmented organisms. The highest estimated percentage of the strain 183BP in the total microbial population was 4.5% on the second day of the October test, based on DAPI-stained total cell counts and real-time PCR analysis of the 183BP population. The percentage of strain 183BP in the total microbial population dropped off rapidly after day 2 and varied

from 0.005% to 0.12% with an average of about 0.04%. The bacterial community in the groundwater of well SE0.5 was alternately dominated by two peaks, a 277-bp fragment that dominated the community profile from the beginning of bacterial and chemical amendment to about the time of greatest CAH removal efficiencies, followed by dominance of a 126-bp fragment for the duration of the tests. This pattern of community development was found to be very similar for both the October and December tests. Cluster analysis of community T-RFLP profiles showed a clear time-dependence for community transition most likely due to natural microbial succession resulting from adaptation to chemical amendment rather than from bioaugmentation, since the major bioaugmented species were not found to dominate the microbial community.

Microbial samples taken from monitoring wells along the flowpath in the bioaugmented well leg clearly show transport through the field of bioaugmented strain 183BP. Real-time PCR results show a 1- to 3-log-order increase in strain 183BP cell concentrations in monitoring wells up to 2 m from the injection well within one day of bioaugmentation. The highest concentration of strain 183BP was found in well SE0.5 with $(6.78\pm0.62)\times10^4$ cells/ml detected 0.4 days after bioaugmentation. Strain 183BP concentrations decreased with distance from the injection well. Although strain 183BP was detected in samples taken from the SE3 monitoring well, they were below the quantifiable detection limit.

In the soil column, maximum treatment efficiencies for 1,1,1-TCA and 1,1-DCE were approximately 96% and 77%, respectively. High influent concentrations of 1,1,1-TCA caused a decrease in removal efficiencies, although on a mass basis more TCA was transformed at higher concentration. Similar to the field observations, the presence of 1,1-DCE inhibited the biotransformation of 1,1,1-TCA. Microbial results indicated that the decline of effluent TCA concentrations was concomitant to the increase in concentration of strain 183BP

cells. The bacterial community had greater species diversity than field samples and less consistent structure over time.

The ability to detect and quantify bioaugmented bacterial strains amidst native communities in environmental samples should lead to a better understanding of aerobic cometabolic transformation of CAHs in natural and engineered systems. In this study, a mixed culture was bioaugmented to an aquifer zone that previously showed no evidence of 1,1,1-TCA transformation ability. Bioaugmentation imparted the desired function of 1,1,1-TCA transformation ability, but the bioaugmented organisms were not found to dominate the microbial community in groundwater samples. A repeatable succession of microbial community profiles was observed in both bioaugmentation tests, presumably as a result of chemical stimulation with butane and oxygen, rather than from bioaugmentation. However, limited microbial analysis of the non-bioaugmented control well leg was insufficient to determine if a similar succession occurred in the control leg. Microbial community profiles at the beginning and ends of the tests indicated that although certain organisms appeared to present in both legs, significantly different populations were developed in the two well legs even though chemical stimulation was the same for both. Hopefully, through the continued application of molecular-based approaches to microbial community structure analysis and in situ population and activity quantification our understanding of complex microbial systems will be significantly enhanced.

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APPENDICES

APPENDIX A

T-RLFP PROFILES

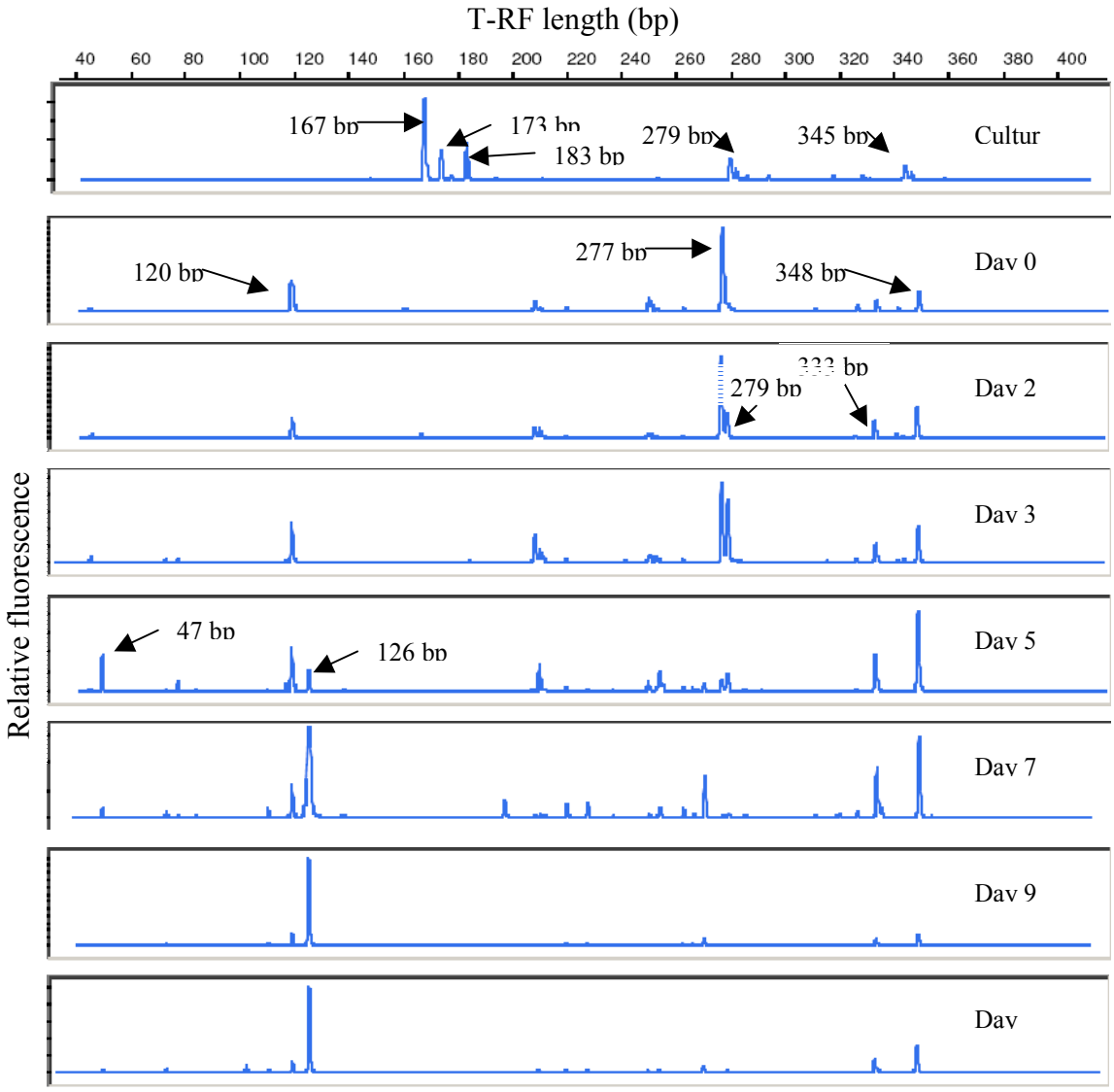


Figure A.1 T-RFLP profiles generated from the bioaugmentation culture grown in the lab and groundwater samples from well SE0.5 during the course of bioaugmentation in October 2003. Universal bacterial primers (27F-B-FAM and 338Rpl) were used in the PCR reactions and the restrictions were performed with the endonuclease *Mnl*I (Fermentas, Inc).

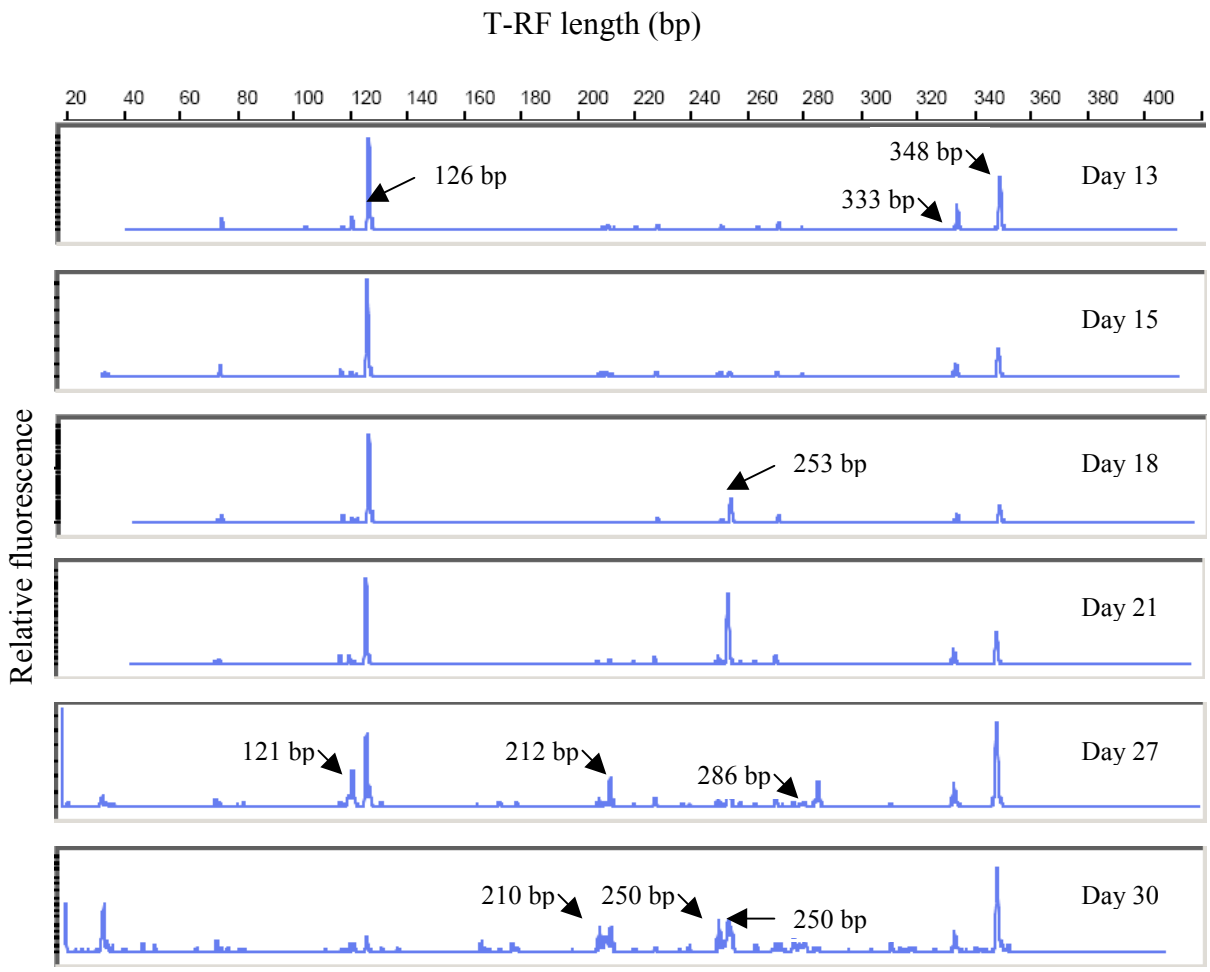


Figure A.1 (Continued) T-RFLP profiles generated from the bioaugmentation culture grown in the lab and groundwater samples from well SE0.5 during the course of bioaugmentation in October 2003. Universal bacterial primers (27F-B-FAM and 338Rpl) were used in the PCR reactions and the restrictions were performed with the endonuclease *Mnl*I (Fermentas, Inc).

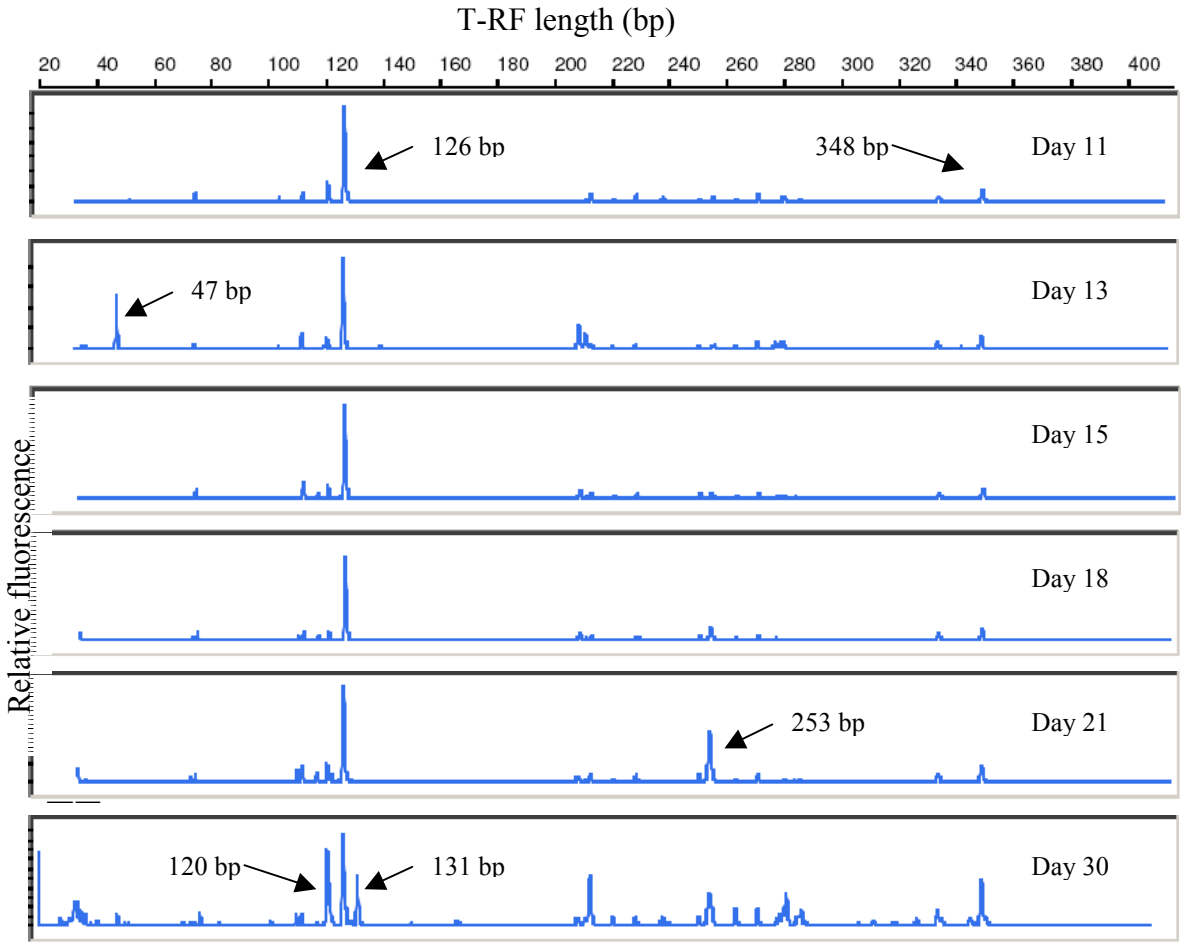


Figure A.2 T-RFLP profiles generated from the bioaugmentation culture grown in the lab and groundwater samples from well SE1 during the course of bioaugmentation in October 2003. Universal bacterial primers (27F-B-FAM and 338Rpl) were used in the PCR reactions and the restrictions were performed with the endonuclease *Mnl*I (Fermentas, Inc).

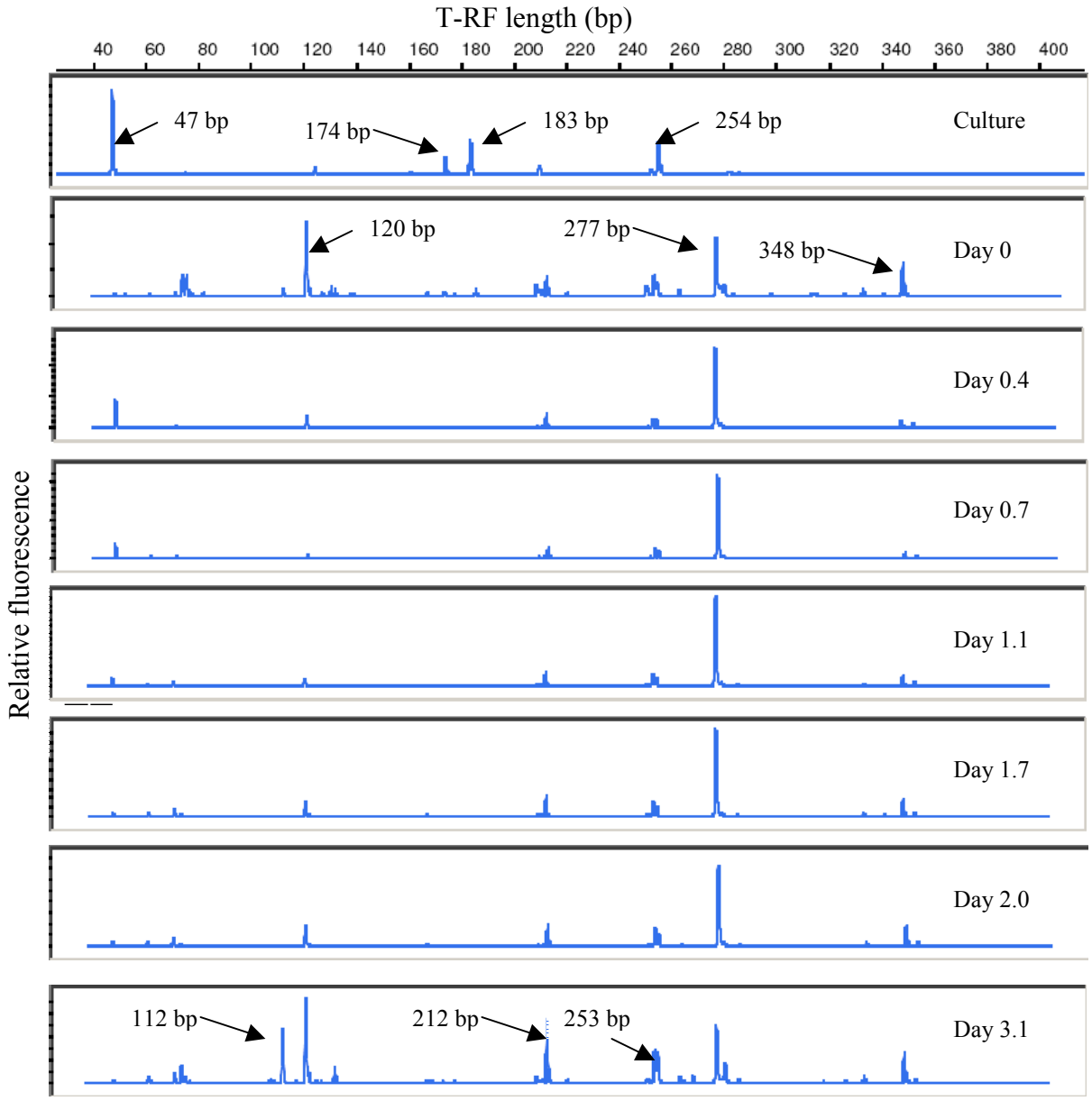


Figure A.3 T-RFLP profiles generated from the bioaugmentation culture grown in the lab and groundwater samples from well SE0.5 during the course of bioaugmentation in December 2003. Universal bacterial primers (27F-B-FAM and 338Rpl) were used in the PCR reactions and the restrictions were performed with the endonuclease *Mnl*I (Fermentas, Inc).

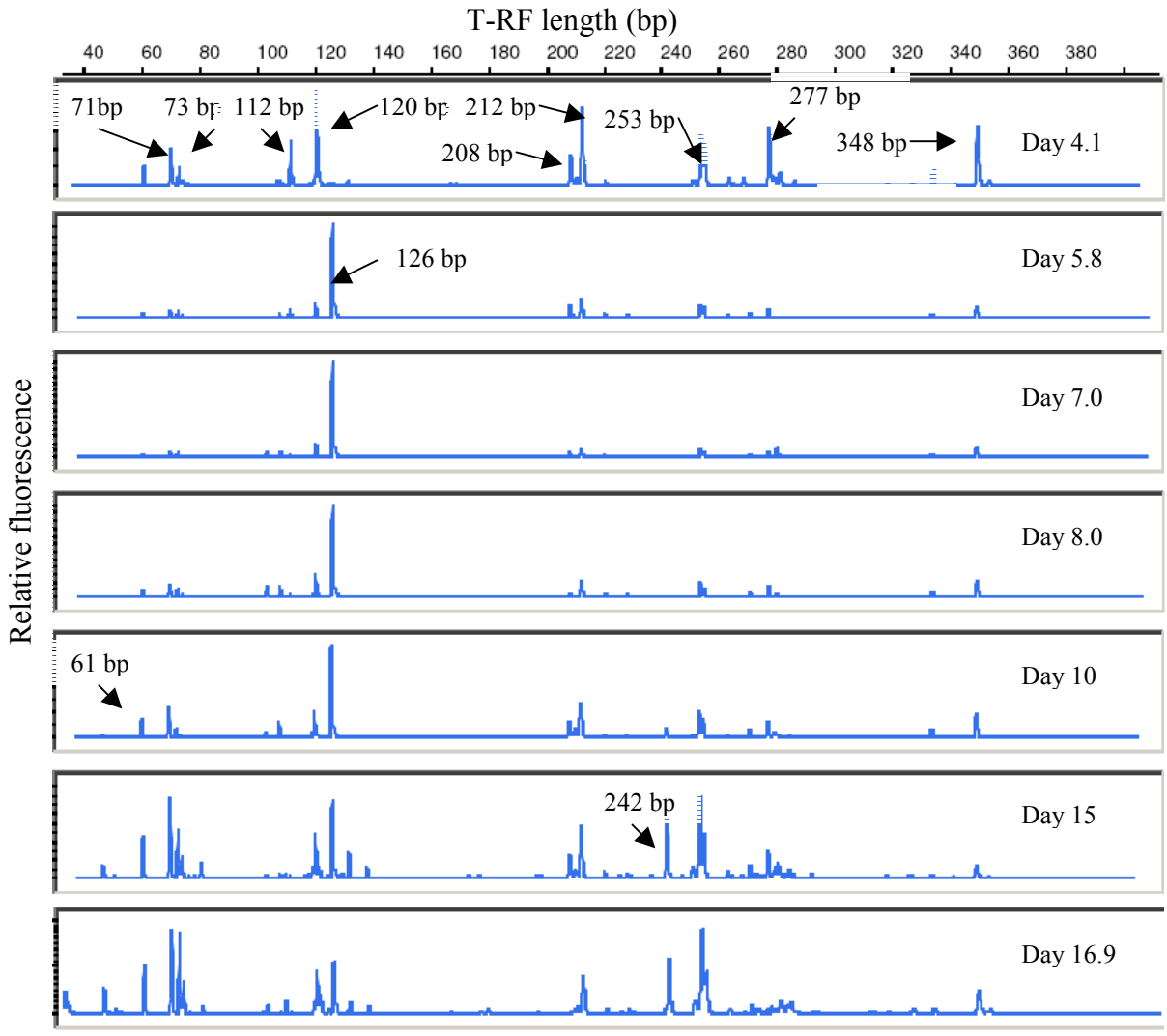


Figure A.3 (Continued) T-RFLP profiles generated from the bioaugmentation culture grown in the lab and groundwater samples from well SE0.5 during the course of bioaugmentation in December 2003. Universal bacterial primers (27F-B-FAM and 338Rpl) were used in the PCR reactions and the restrictions were performed with the endonuclease *Mnl*I (Fermentas, Inc).

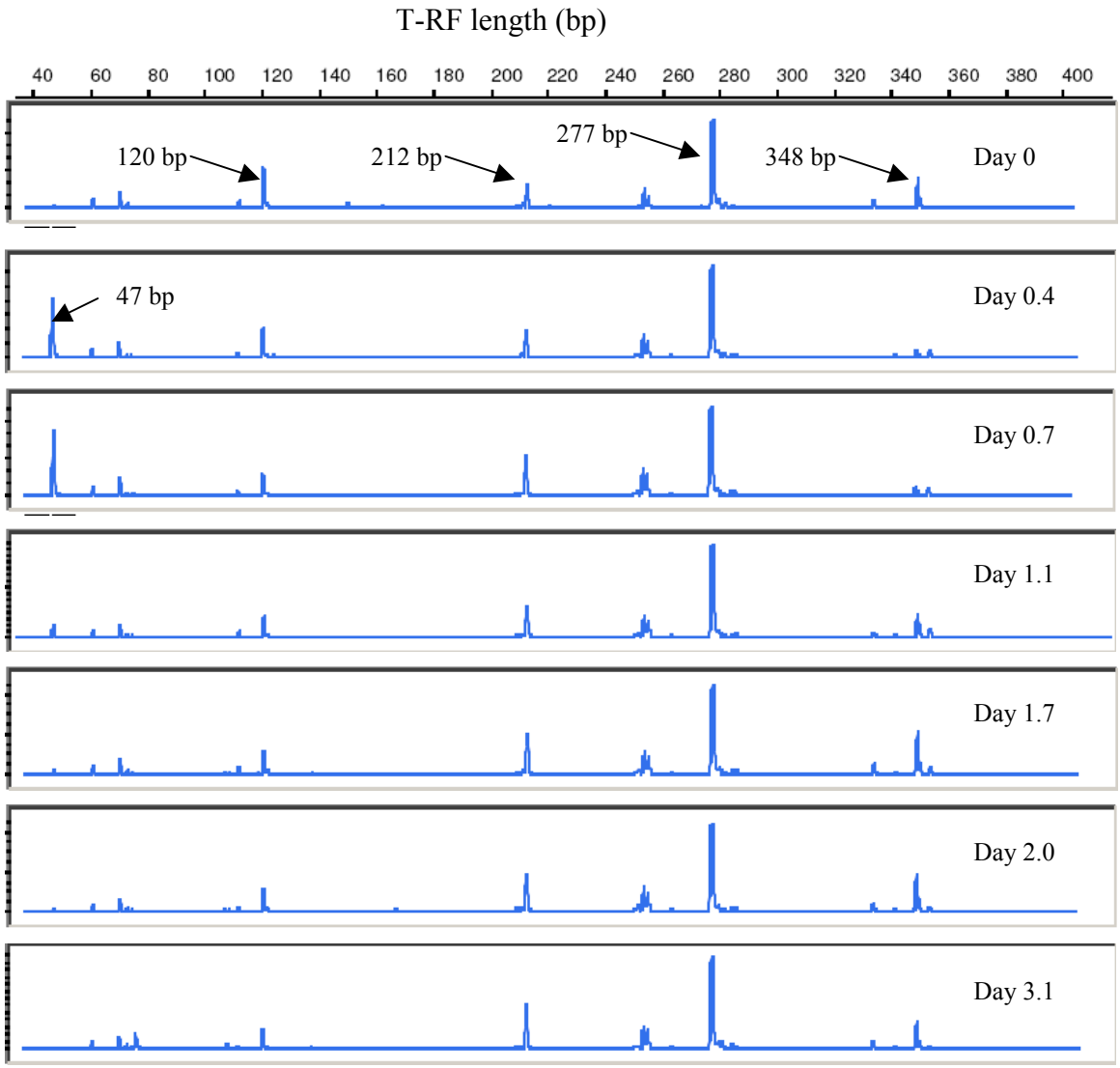


Figure A.4 T-RFLP profiles generated from the bioaugmentation culture grown in the lab and groundwater samples from well SE1 during the course of bioaugmentation in December 2003. Universal bacterial primers (27F-B-FAM and 338Rpl) were used in the PCR reactions and the restrictions were performed with the endonuclease *Mnl*I (Fermentas, Inc).

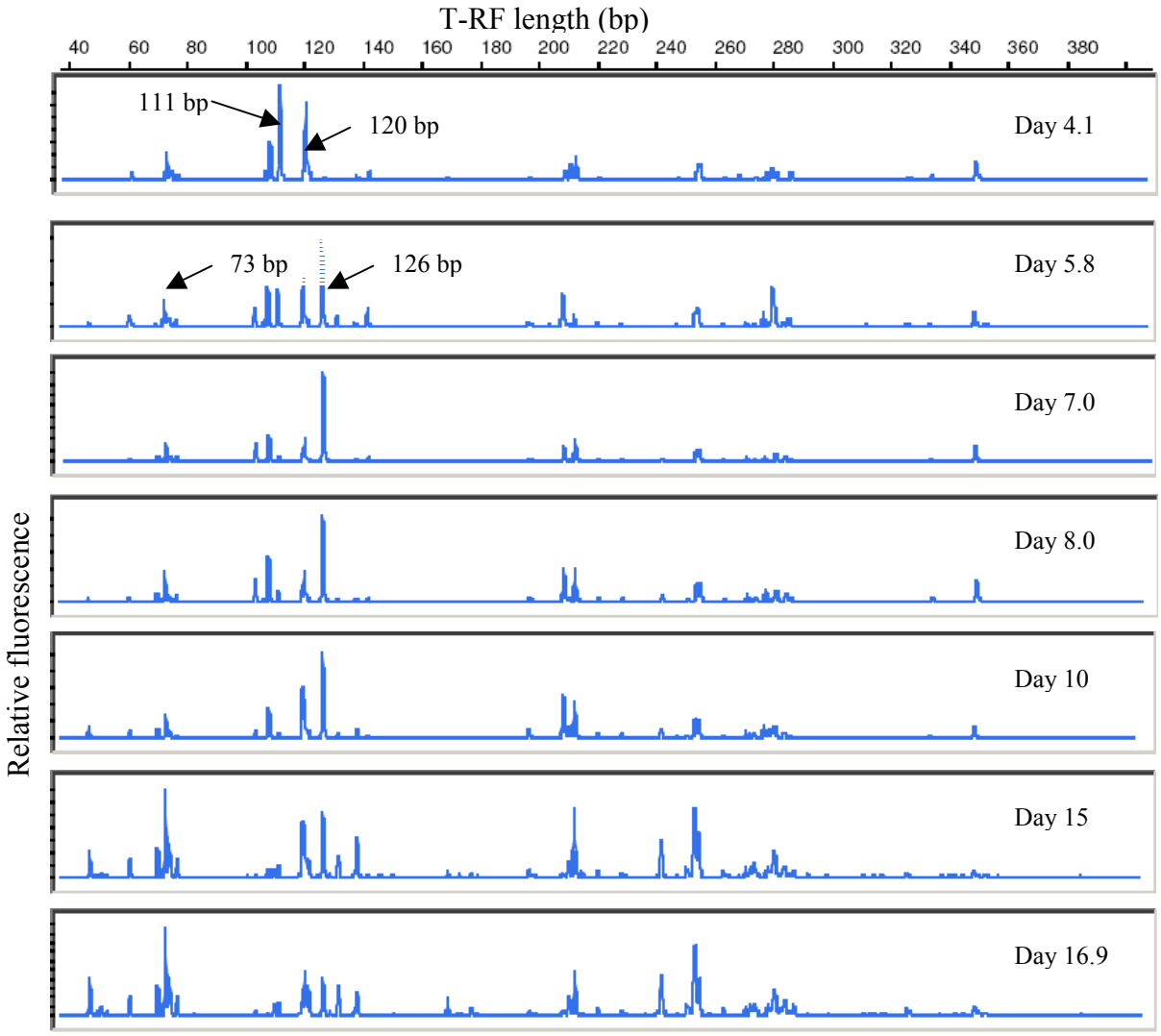


Figure A.4 (Continued) T-RFLP profiles generated from the bioaugmentation culture grown in the lab and groundwater samples from well SE0.5 during the course of bioaugmentation in December 2003. Universal bacterial primers (27F-B-FAM and 338Rpl) were used in the PCR reactions and the restrictions were performed with the endonuclease *Mnl*I (Fermentas, Inc).

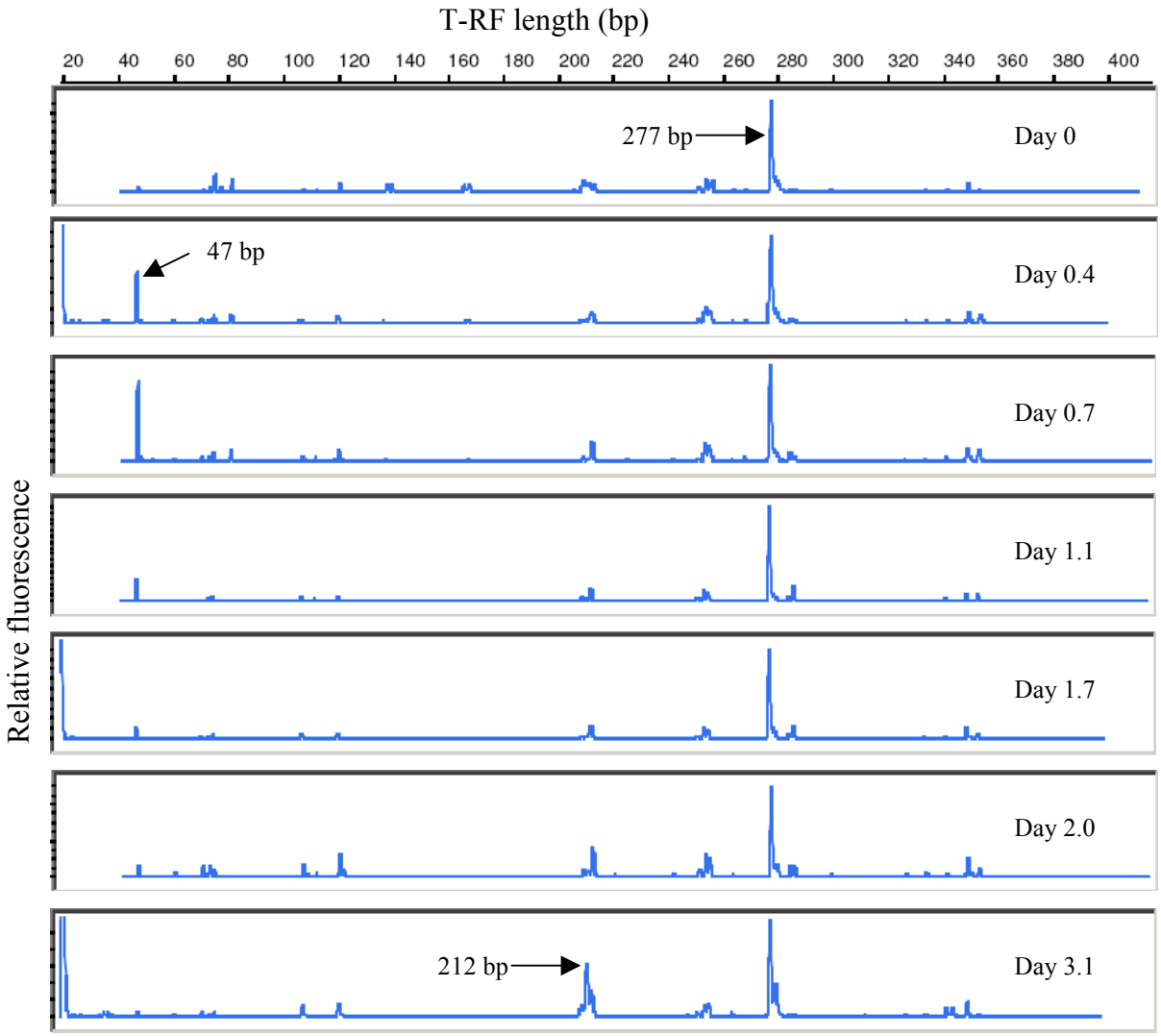


Figure A.5 T-RFLP profiles generated from the bioaugmentation culture grown in the lab and groundwater samples from well SE1.5 during the course of bioaugmentation in December 2003. Universal bacterial primers (27F-B-FAM and 338Rpl) were used in the PCR reactions and the restrictions were performed with the endonuclease *Mnl*I (Fermentas, Inc).

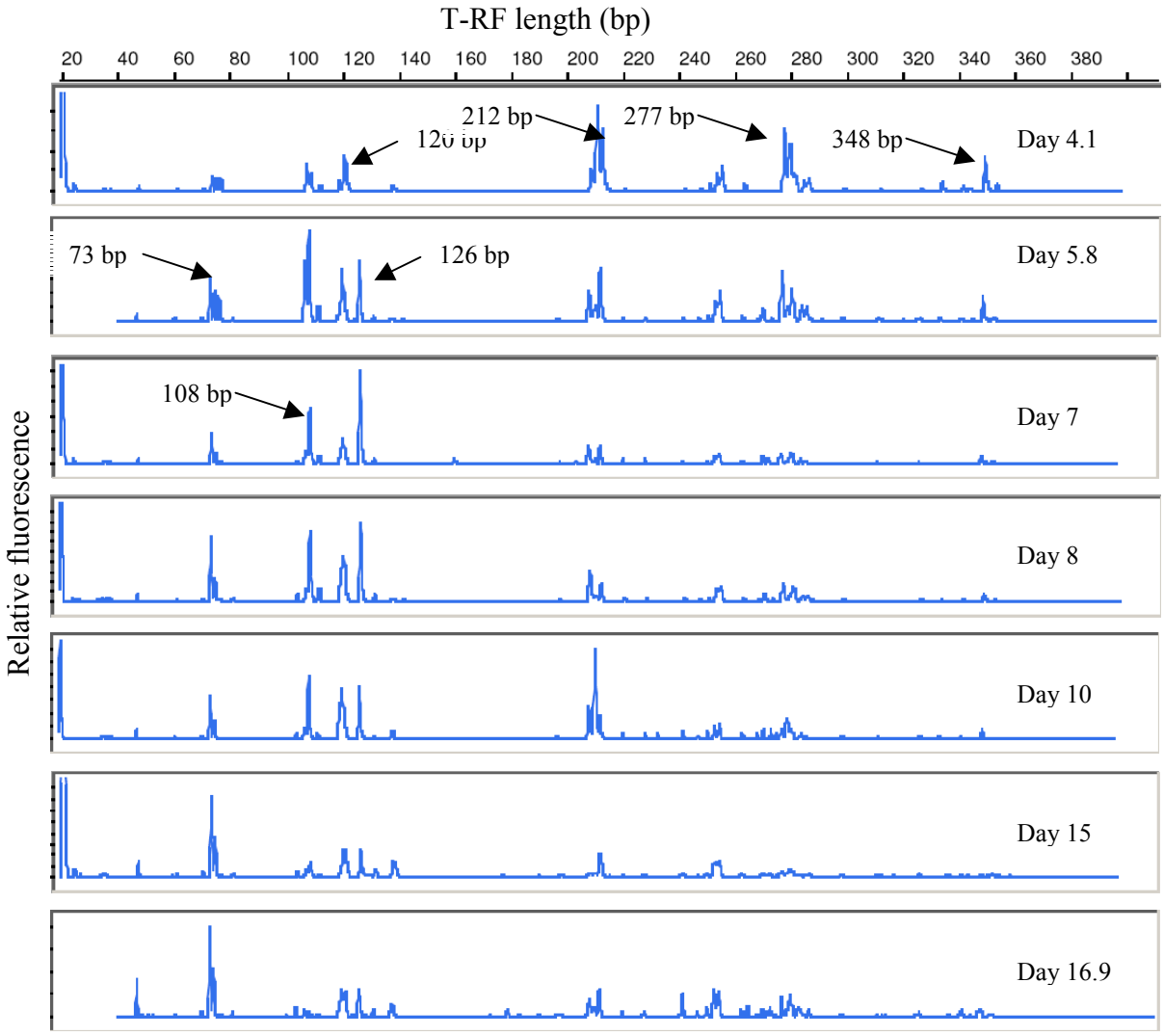


Figure A.5 (Continued) T-RFLP profiles generated from the bioaugmentation culture grown in the lab and groundwater samples from well SE1.5 during the course of bioaugmentation in December 2003. Universal bacterial primers (27F-B-FAM and 338Rpl) were used in the PCR reactions and the restrictions were performed with the endonuclease *Mnl*I (Fermentas, Inc).

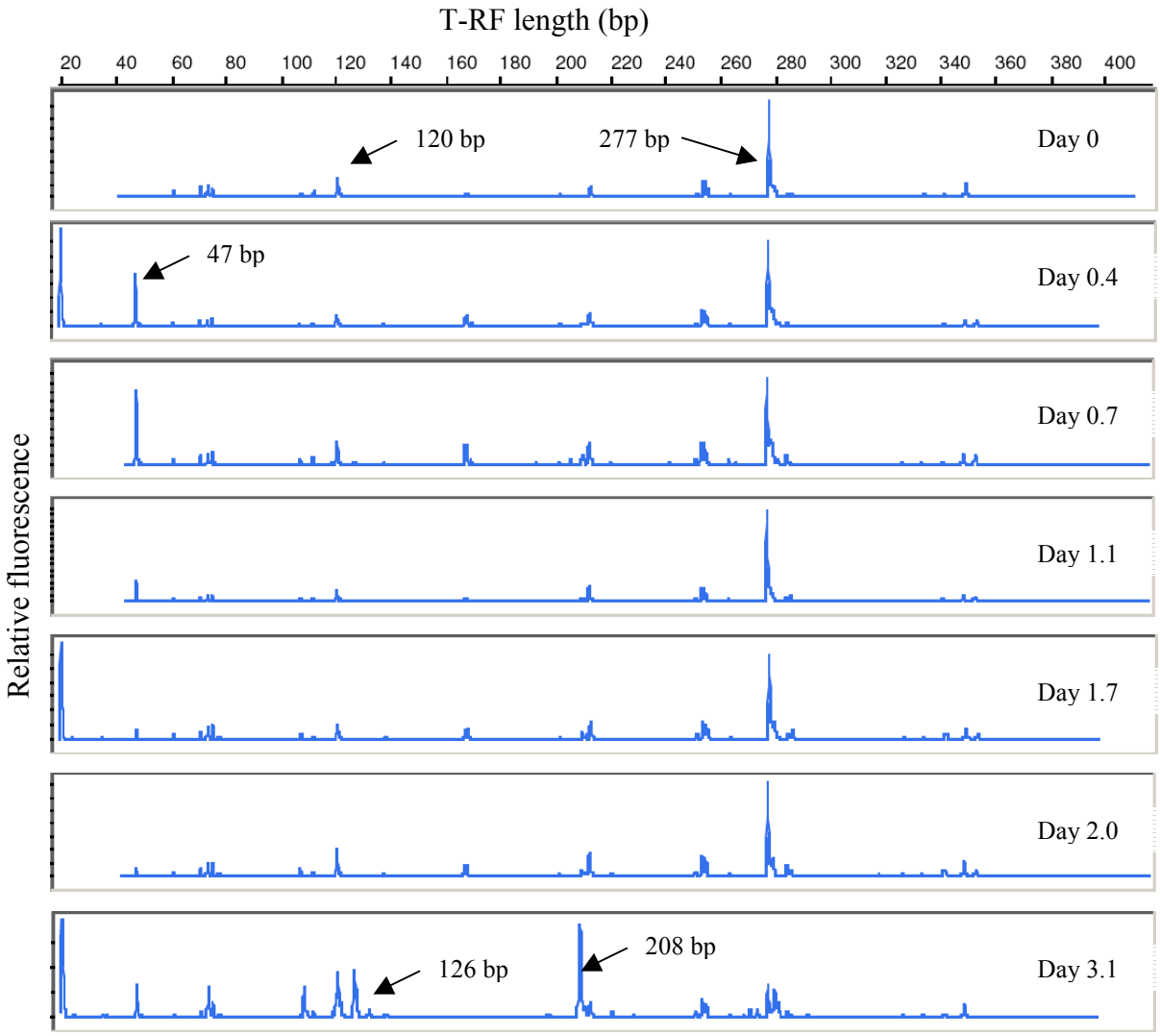


Figure A.6 T-RFLP profiles generated from the bioaugmentation culture grown in the lab and groundwater samples from well SE2 during the course of bioaugmentation in December 2003. Universal bacterial primers (27F-B-FAM and 338Rpl) were used in the PCR reactions and the restrictions were performed with the endonuclease *Mnl*I (Fermentas, Inc).

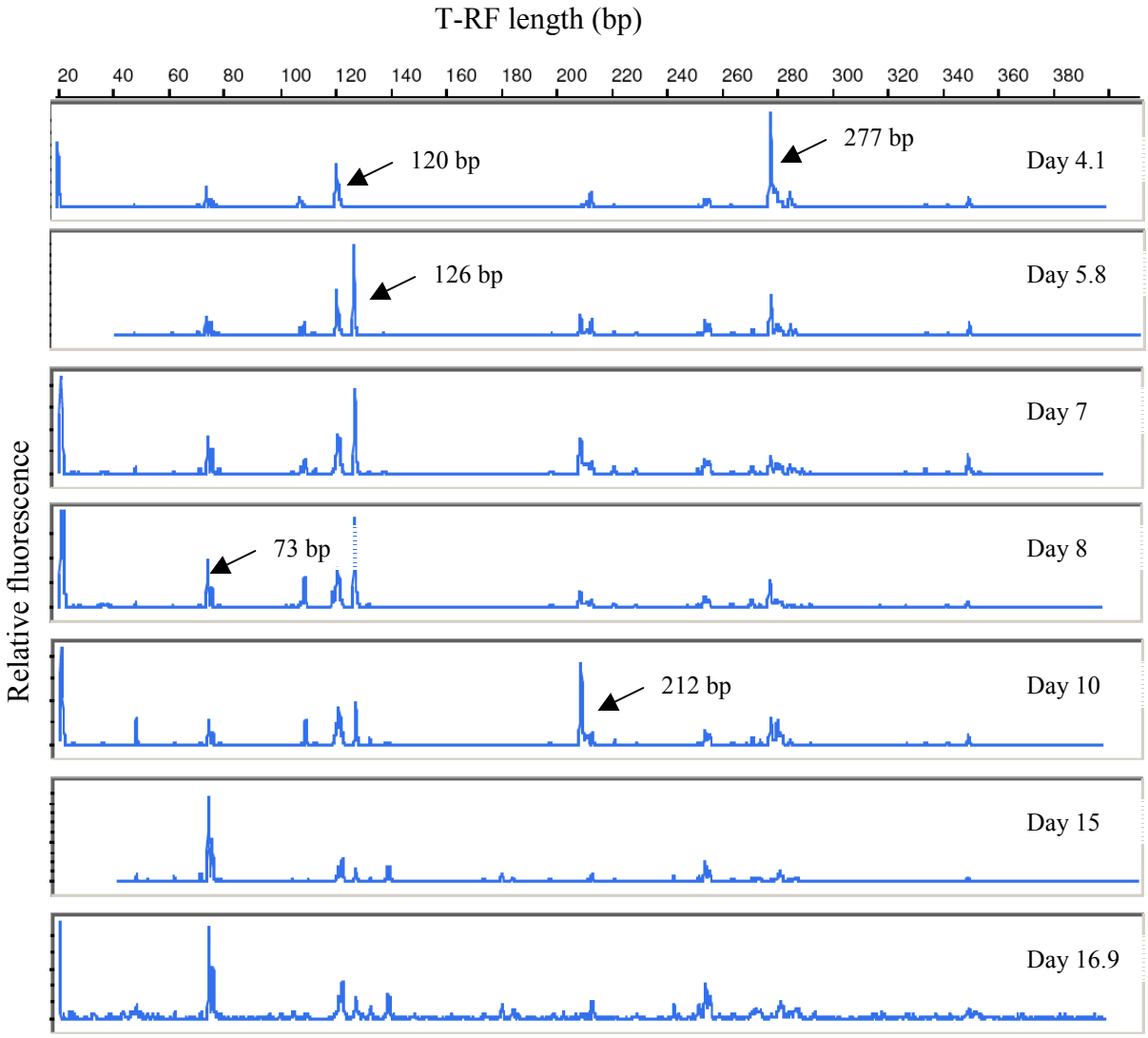


Figure A.6 (Continued) T-RFLP profiles generated from the bioaugmentation culture grown in the lab and groundwater samples from well SE2 during the course of bioaugmentation in December 2003. Universal bacterial primers (27F-B-FAM and 338Rpl) were used in the PCR reactions and the restrictions were performed with the endonuclease *Mnl*I (Fermentas, Inc).

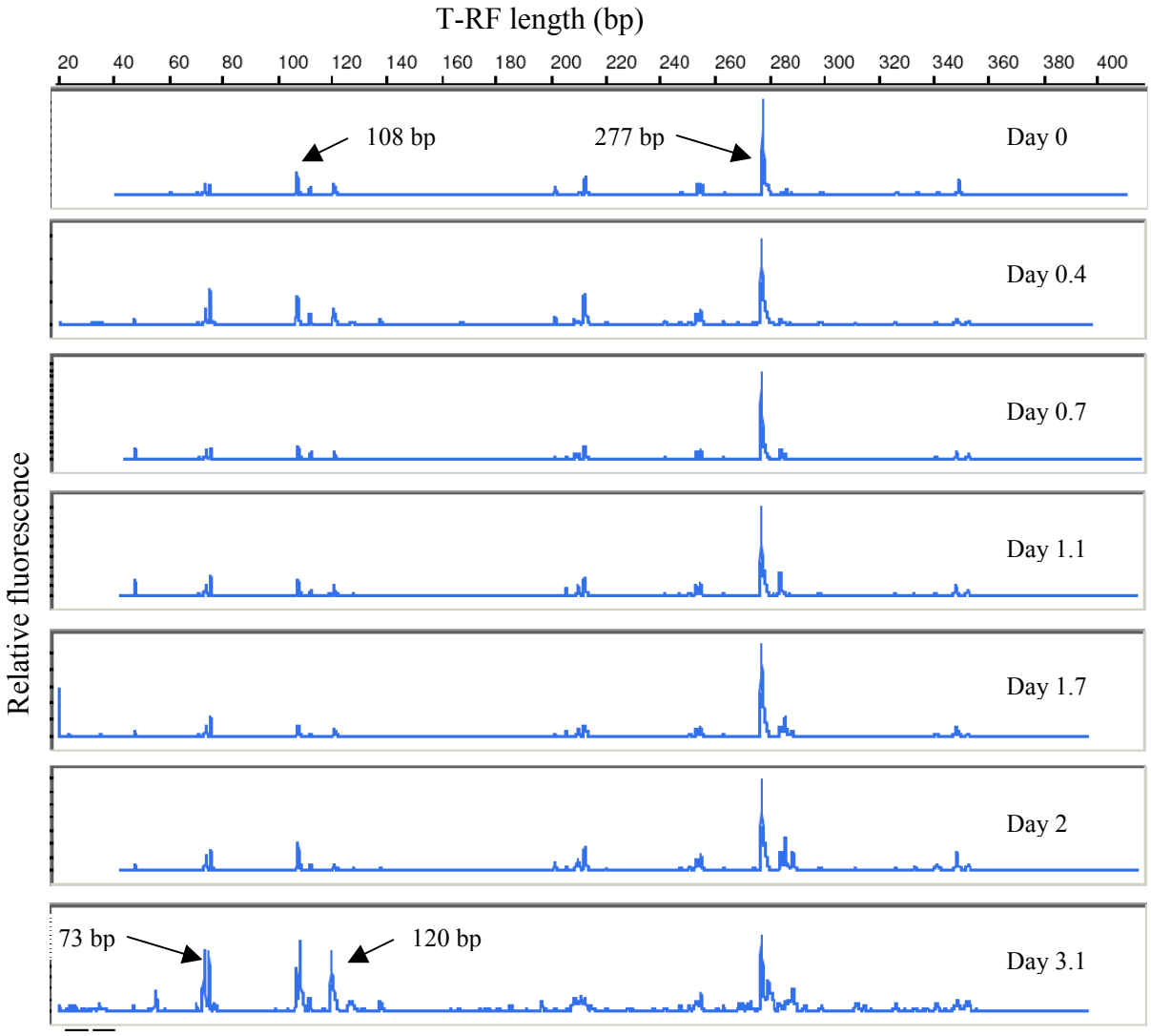


Figure A.7 T-RFLP profiles generated from the bioaugmentation culture grown in the lab and groundwater samples from well SE3 during the course of bioaugmentation in December 2003. Universal bacterial primers (27F-B-FAM and 338Rpl) were used in the PCR reactions and the restrictions were performed with the endonuclease *Mnl*I (Fermentas, Inc).

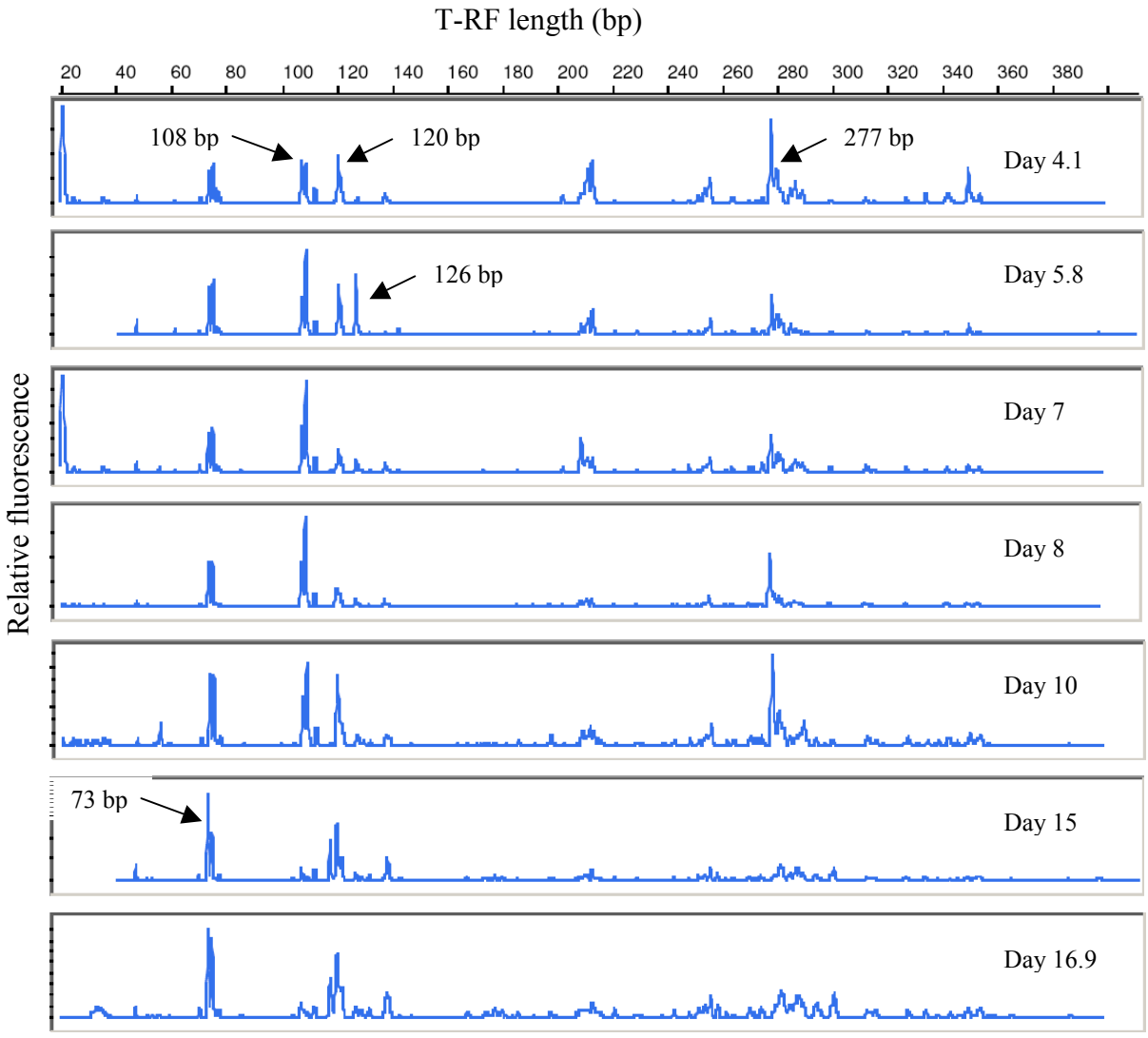


Figure A.7 (Continued) T-RFLP profiles generated from the bioaugmentation culture grown in the lab and groundwater samples from well SE3 during the course of bioaugmentation in December 2003. Universal bacterial primers (27F-B-FAM and 338Rpl) were used in the PCR reactions and the restrictions were performed with the endonuclease *Mnl*I (Fermentas, Inc).

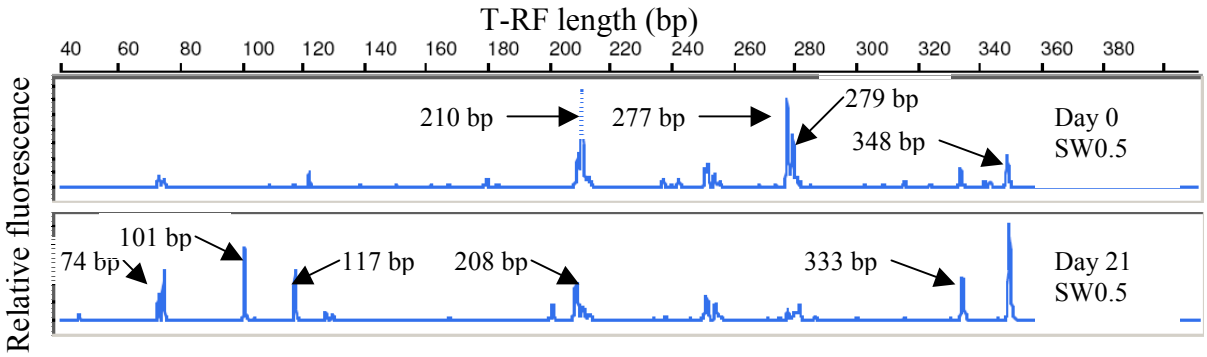


Figure A.8 T-RFLP profiles generated groundwater samples from well SW0.5 during the course of bioaugmentation in October 2003. Universal bacterial primers (27F-B-FAM and 338Rpl) were used in the PCR reactions and the restrictions were performed with the endonuclease *Mnl*I (Fermentas, Inc).

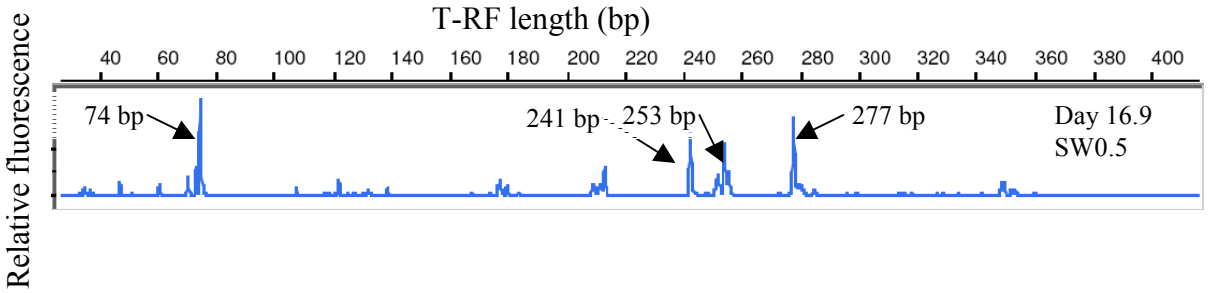


Figure A.9 T-RFLP profiles generated from the groundwater sample from well SW0.5 during the course of bioaugmentation in December 2003. Universal bacterial primers (27F-B-FAM and 338Rpl) were used in the PCR reactions and the restrictions were performed with the endonuclease *Mnl*I (Fermentas, Inc).

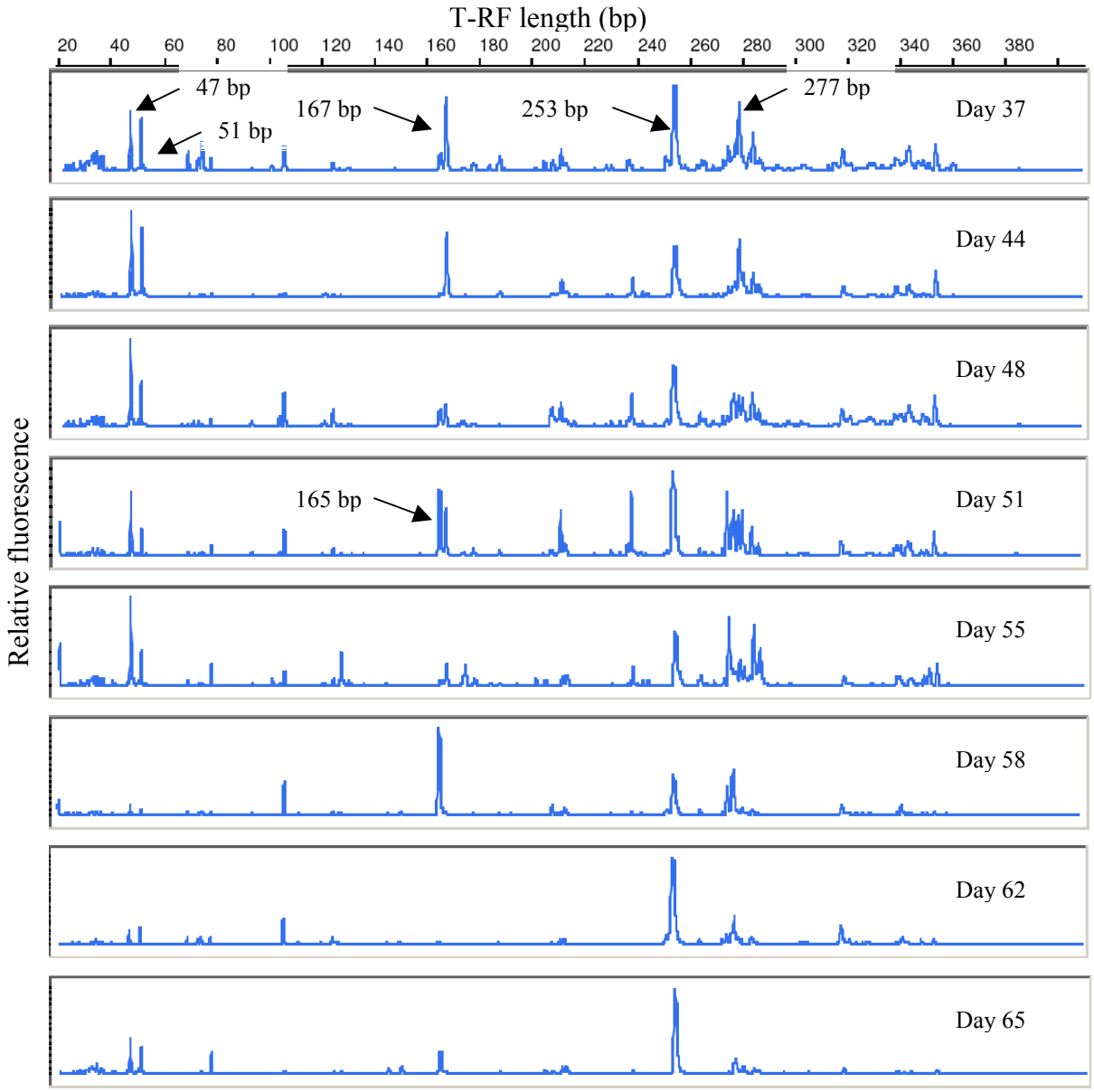


Figure A.10 T-RFLP profiles generated soil column effluent samples during the course of bioaugmentation test. Universal bacterial primers (27F-B-FAM and 338Rpl) were used in the PCR reactions and the restrictions were performed with the endonuclease *Mnl*I (Fermentas, Inc).

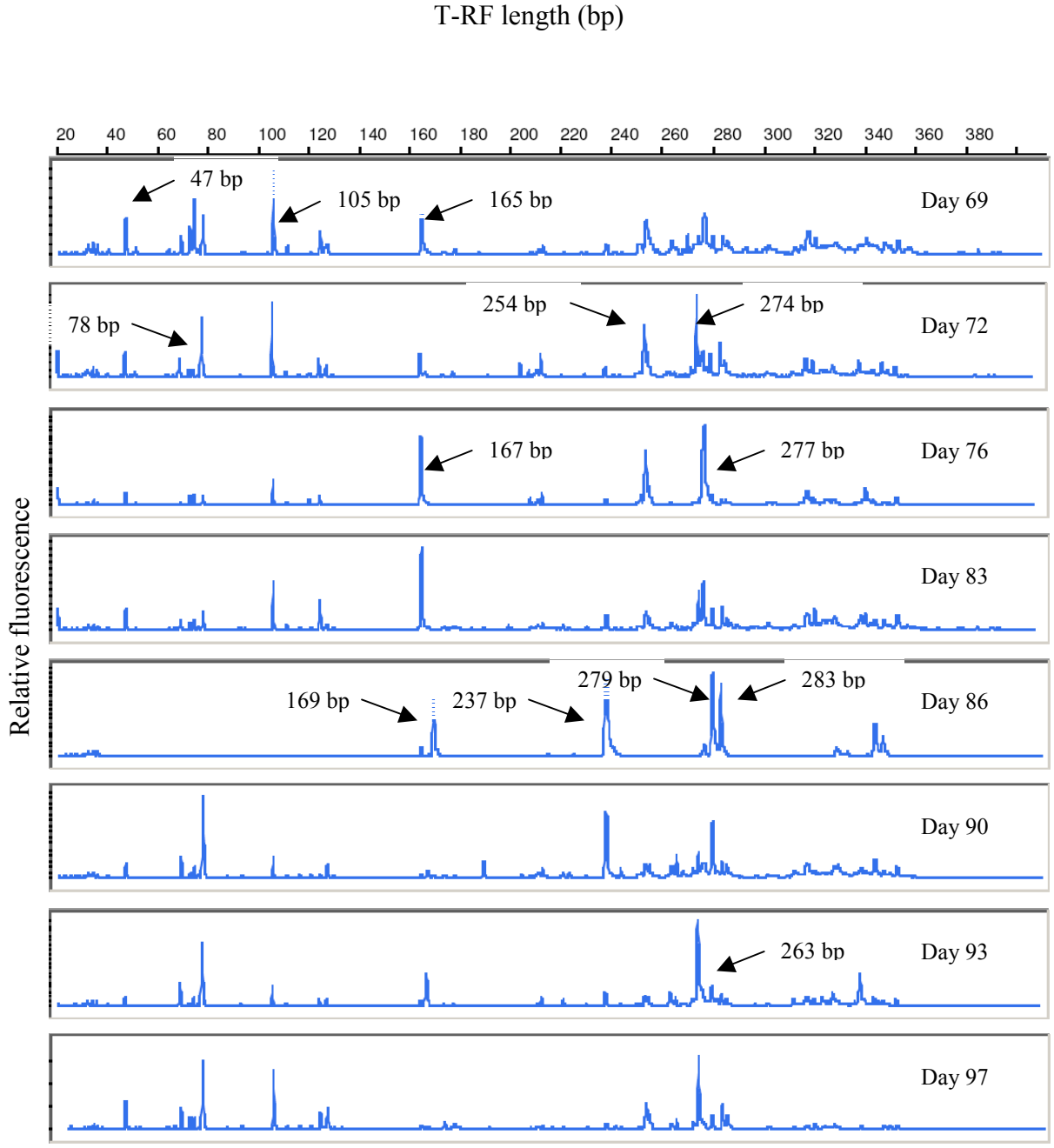


Figure A.10 (Continued) T-RFLP profiles generated soil column effluent samples during the course of bioaugmentation test. Universal bacterial primers (27F-B-FAM and 338Rpl) were used in the PCR reactions and the restrictions were performed with the endonuclease *Mnl*I (Fermentas, Inc).

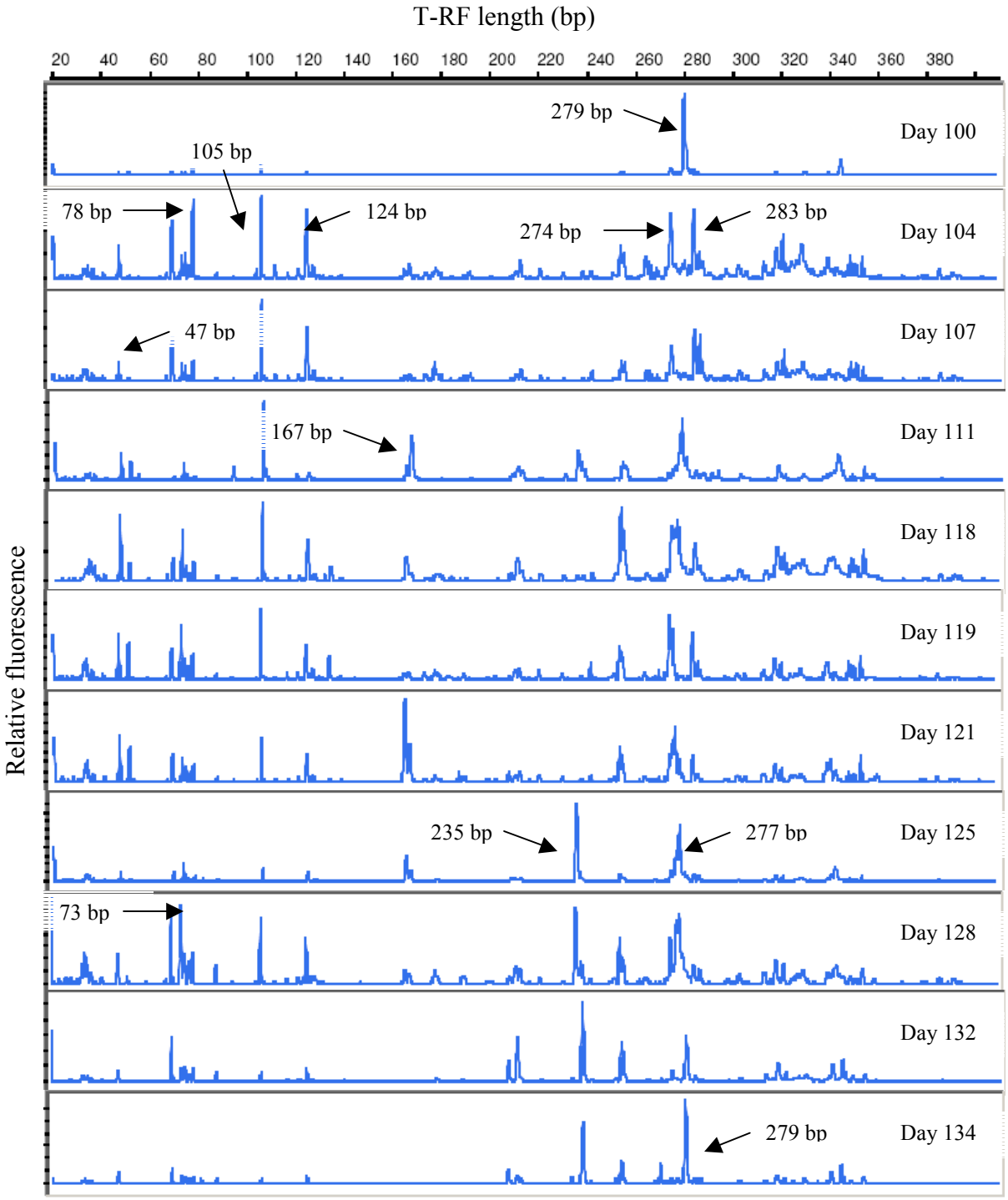


Figure A.10 (Continued) T-RFLP profiles generated soil column effluent samples during the course of bioaugmentation test. Universal bacterial primers (27F-B-FAM and 338Rpl) were used in the PCR reactions and the restrictions were performed with the endonuclease *Mnl*I (Fermentas, Inc).

APPENDIX B

SIMILARITY ANALYSIS OF T-RRFLP PROFILE PATTERNS

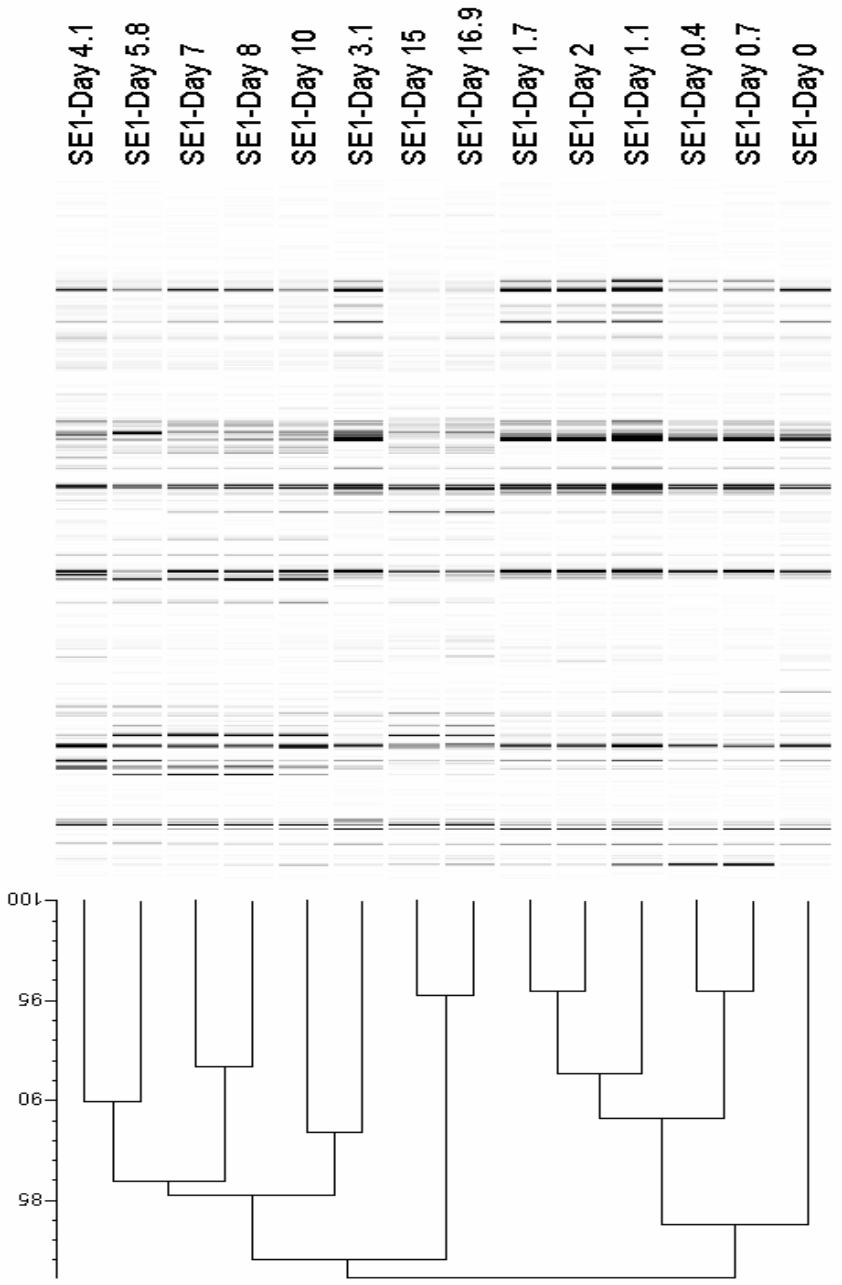


Figure B.1 A GelComparII-generated UPGMA clustering dendrogram and corresponding normalized restriction profiles from the community DNA extracted from the groundwater samples taken form well SE1 in December, 2003.

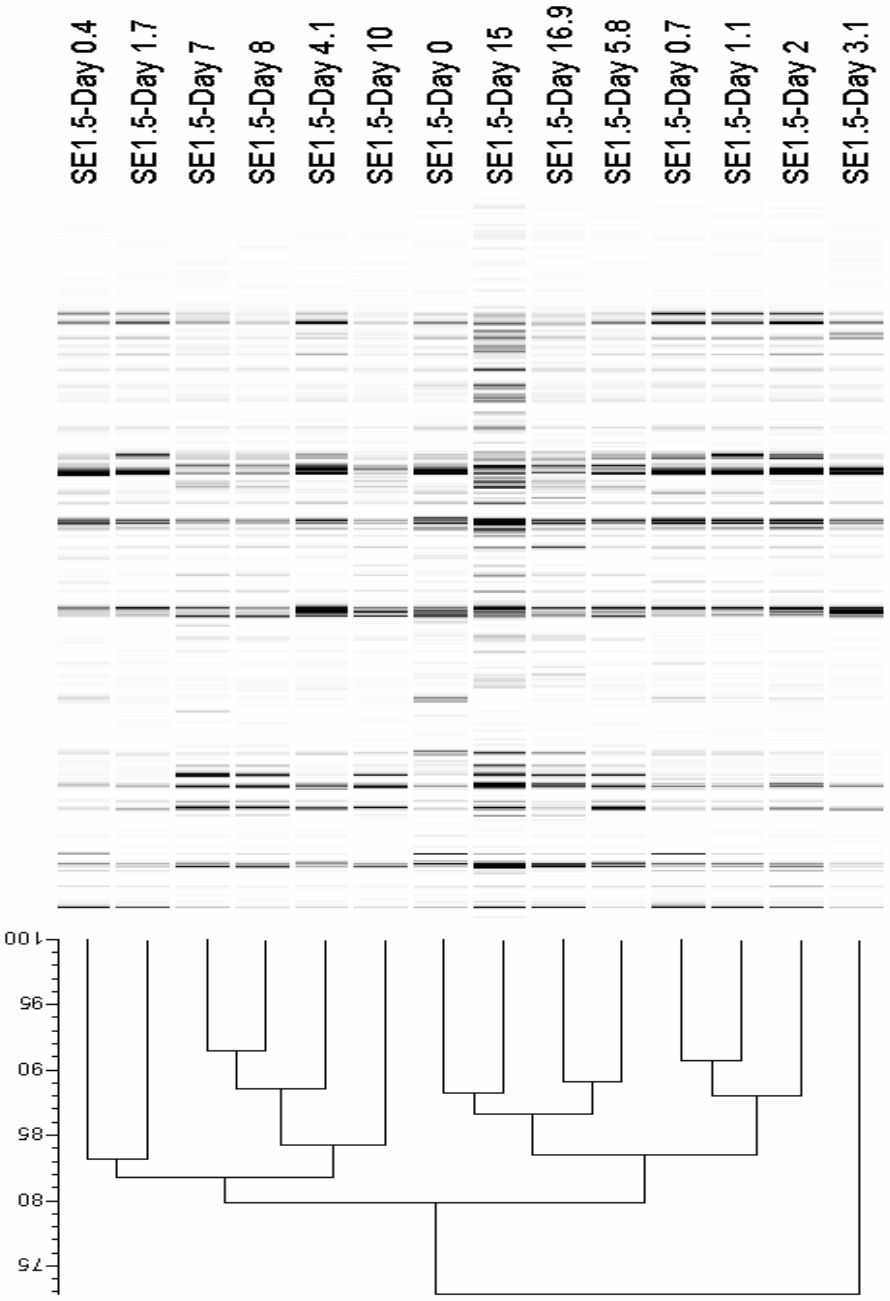


Figure B.2 A GelComparII-generated UPGMA clustering dendrogram and corresponding normalized restriction profiles from the community DNA extracted from the groundwater samples taken form well SE1.5 in December, 2003.

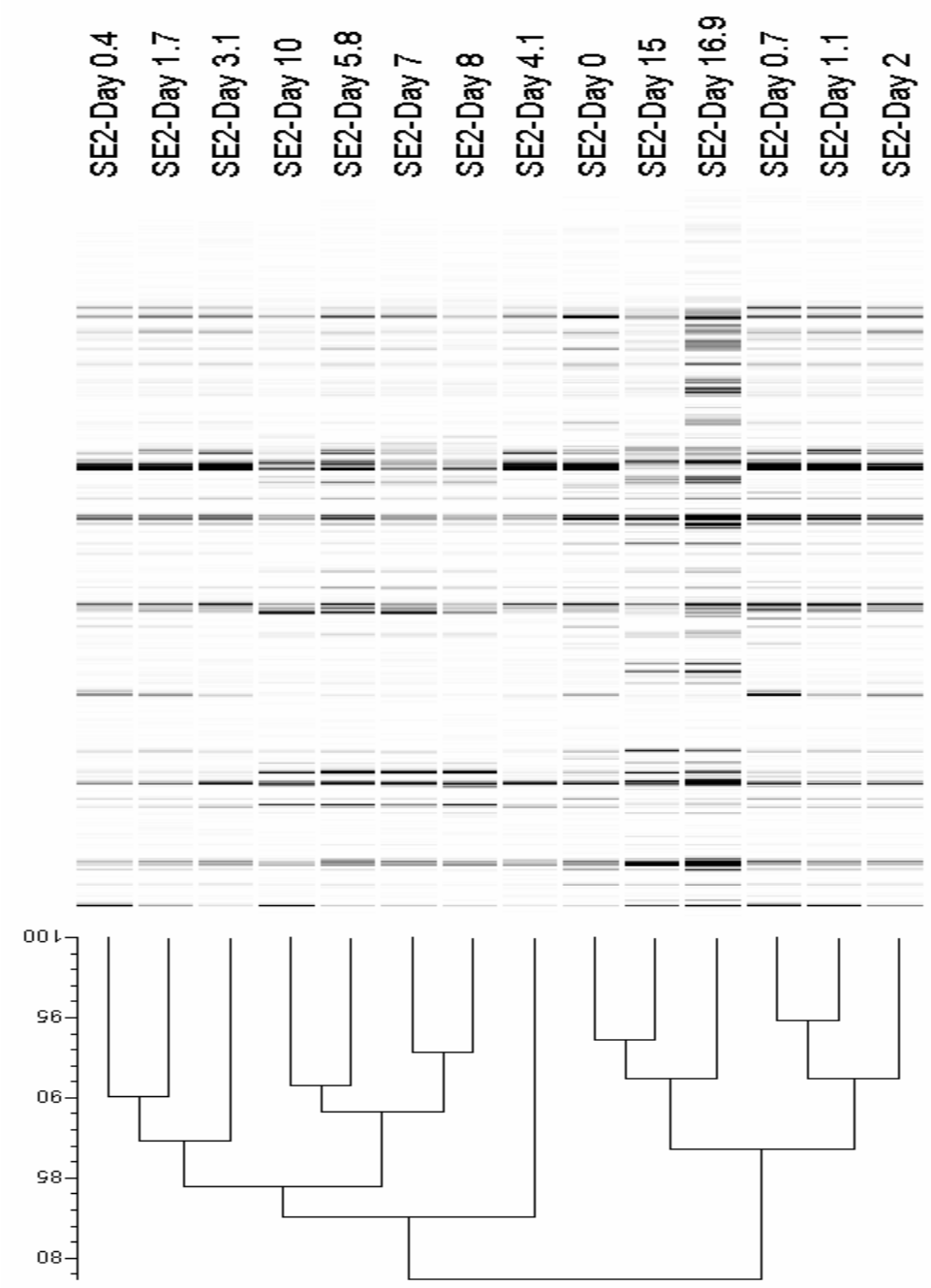


Figure B.3 A GelComparII-generated UPGMA clustering dendrogram and corresponding normalized restriction profiles from the community DNA extracted from the groundwater samples taken form well SE2 in December, 2003.

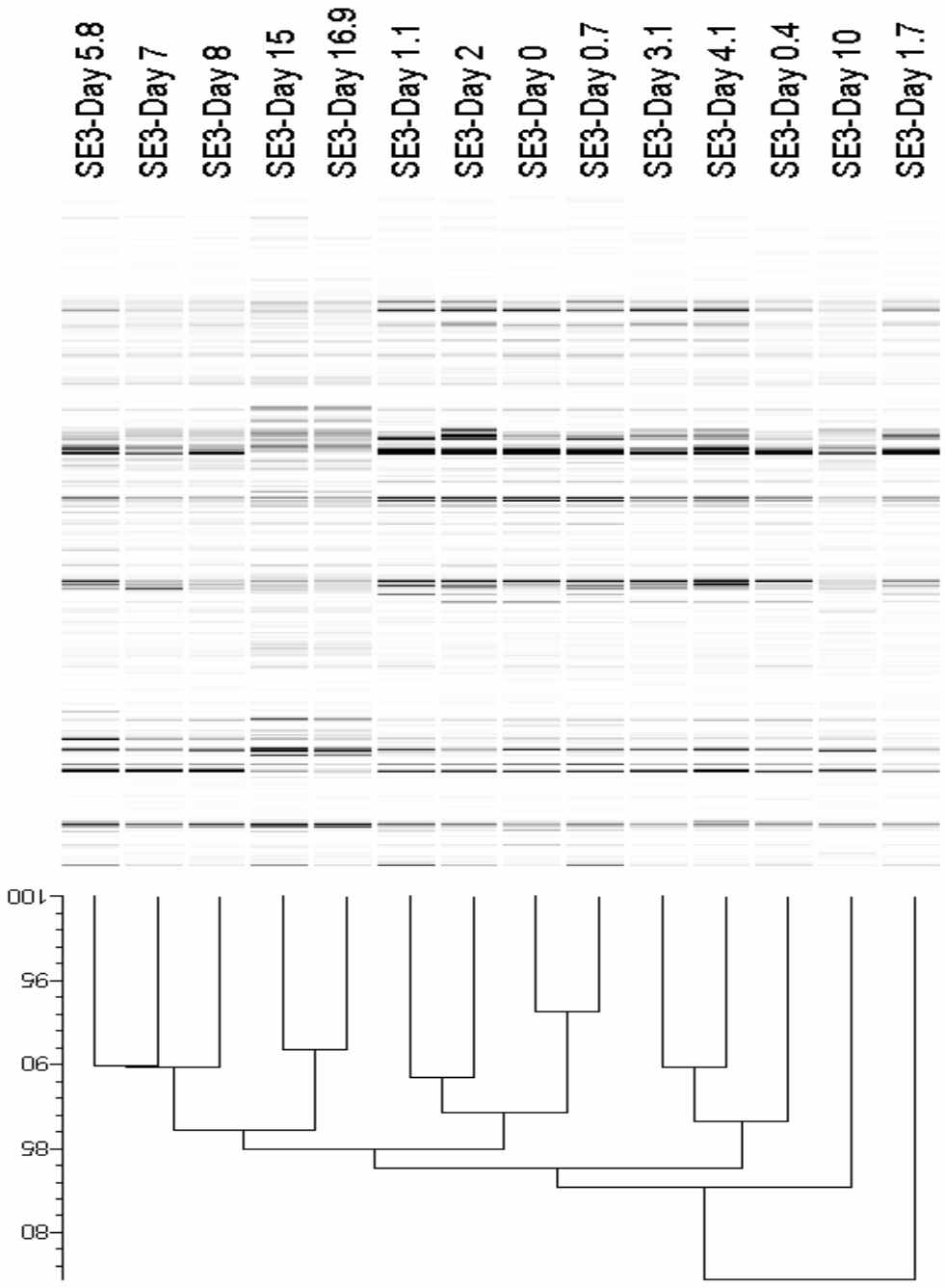


Figure B.4 A GelComparII-generated UPGMA clustering dendrogram and corresponding normalized restriction profiles from the community DNA extracted from the groundwater samples taken form well SE3 in December, 2003.

APPENDIX C

PERCENTAGES OF PEAK AREAS IN T-RLFP PROFILE

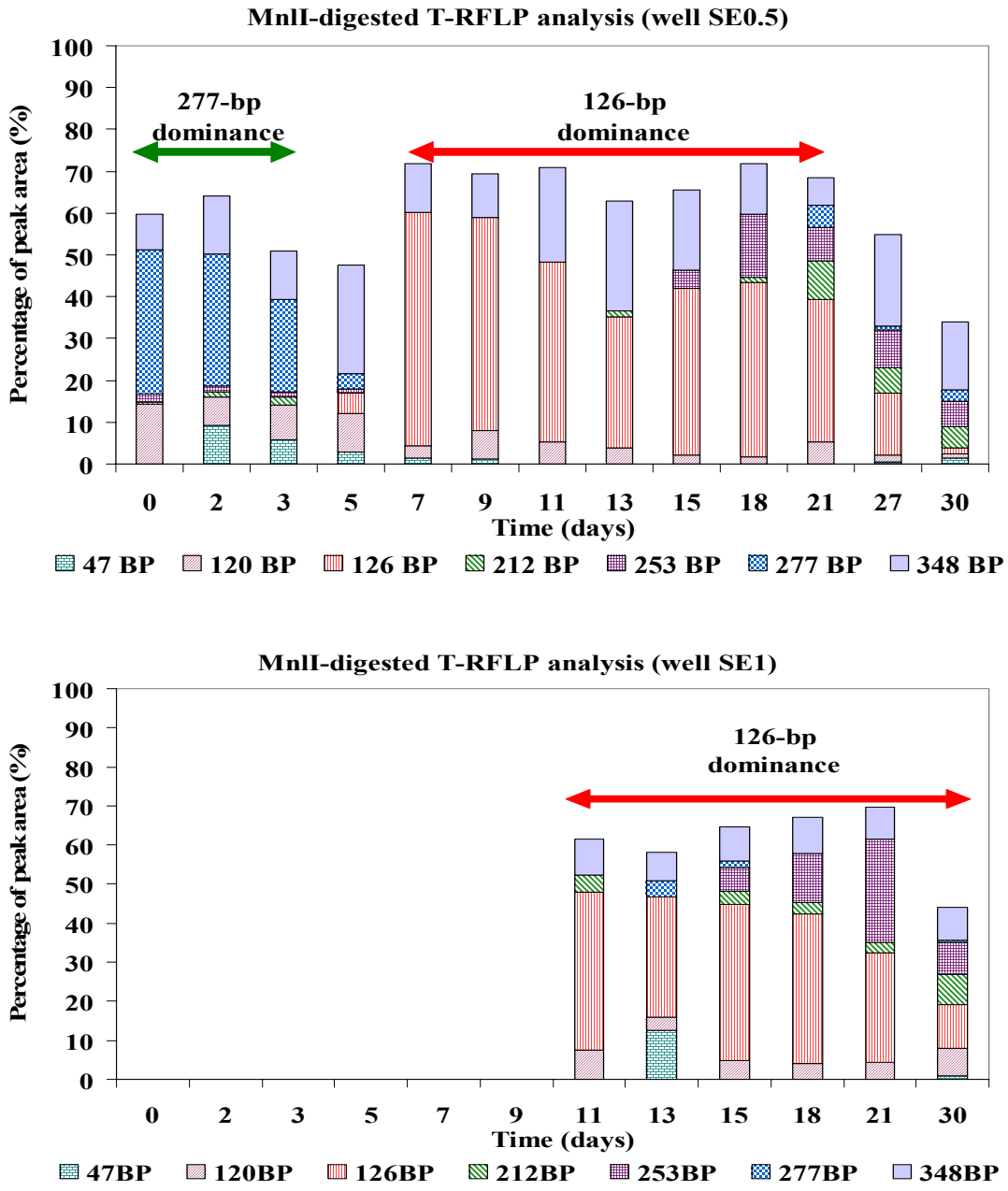


Figure C.1 Changes in the microbial community structure during the bioaugmentation test in well SE0.5 and SE1 during the bioaugmentation test conducted in October, 2003.

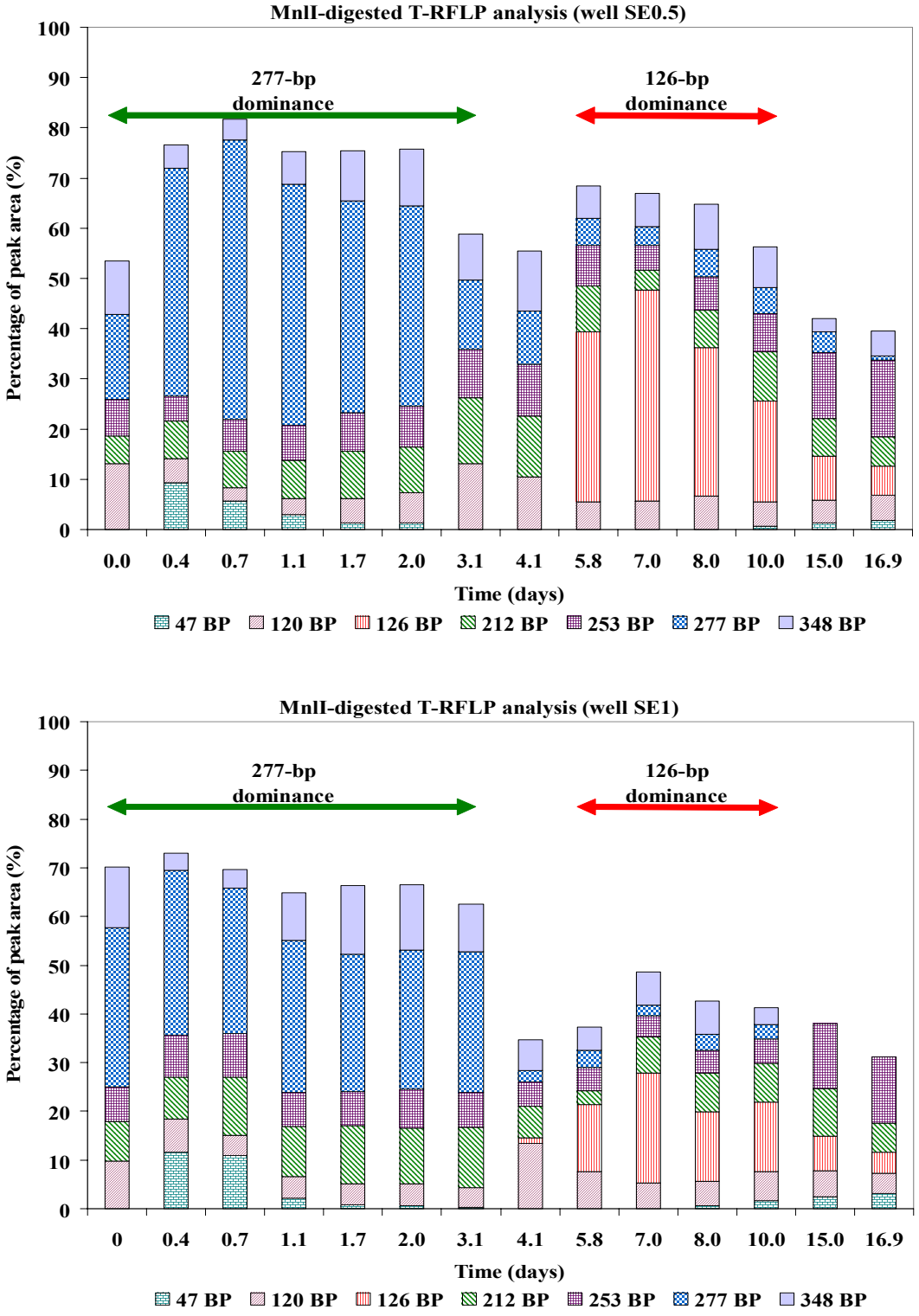


Figure C.2 Changes in the microbial community structure during the bioaugmentation test in well SE0.5 and SE1 during the bioaugmentation test conducted in December, 2003.

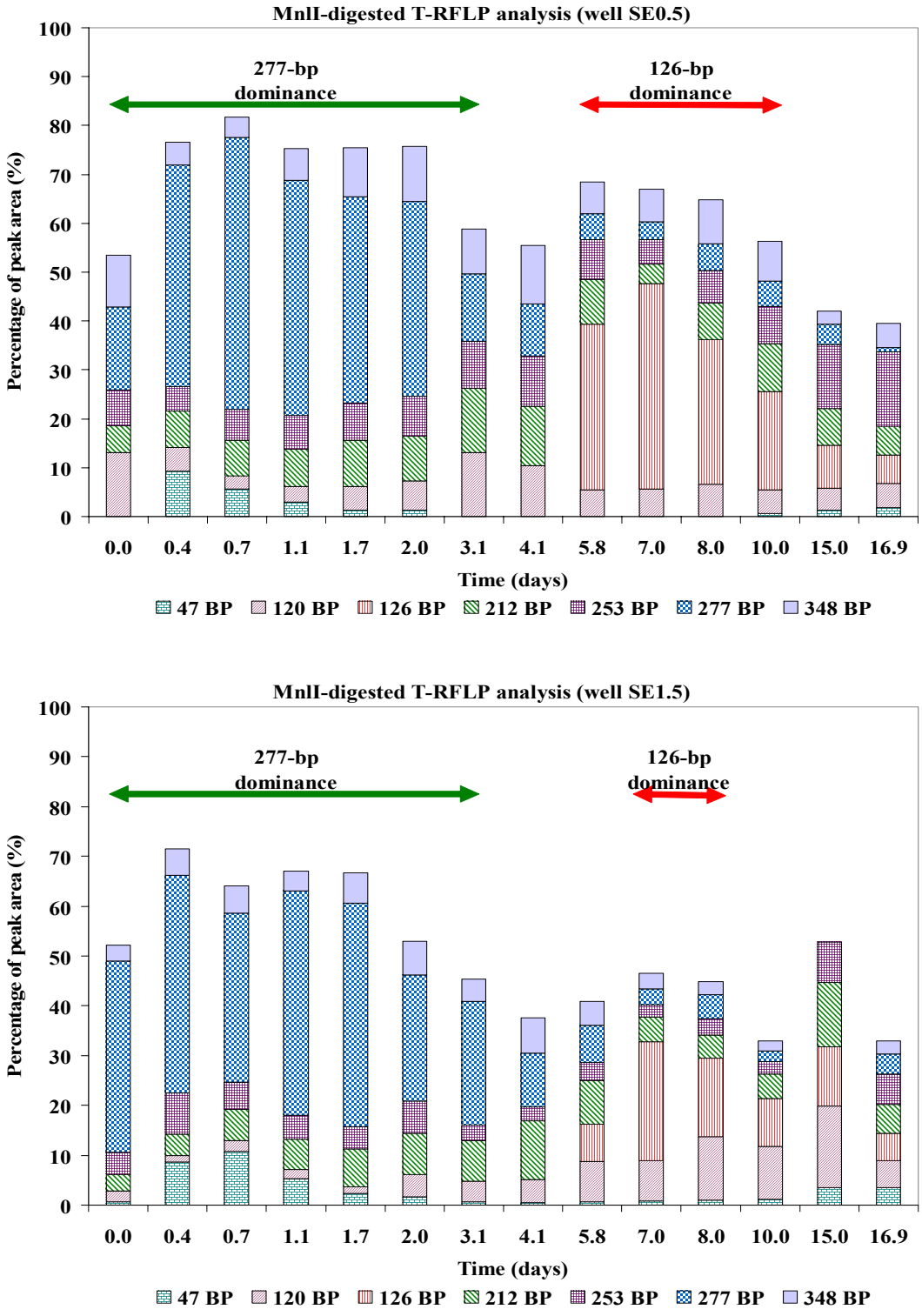


Figure C.3 Changes in the microbial community structure during the bioaugmentation test in well SE0.5 and SE1.5 during the bioaugmentation test conducted in December, 2003.

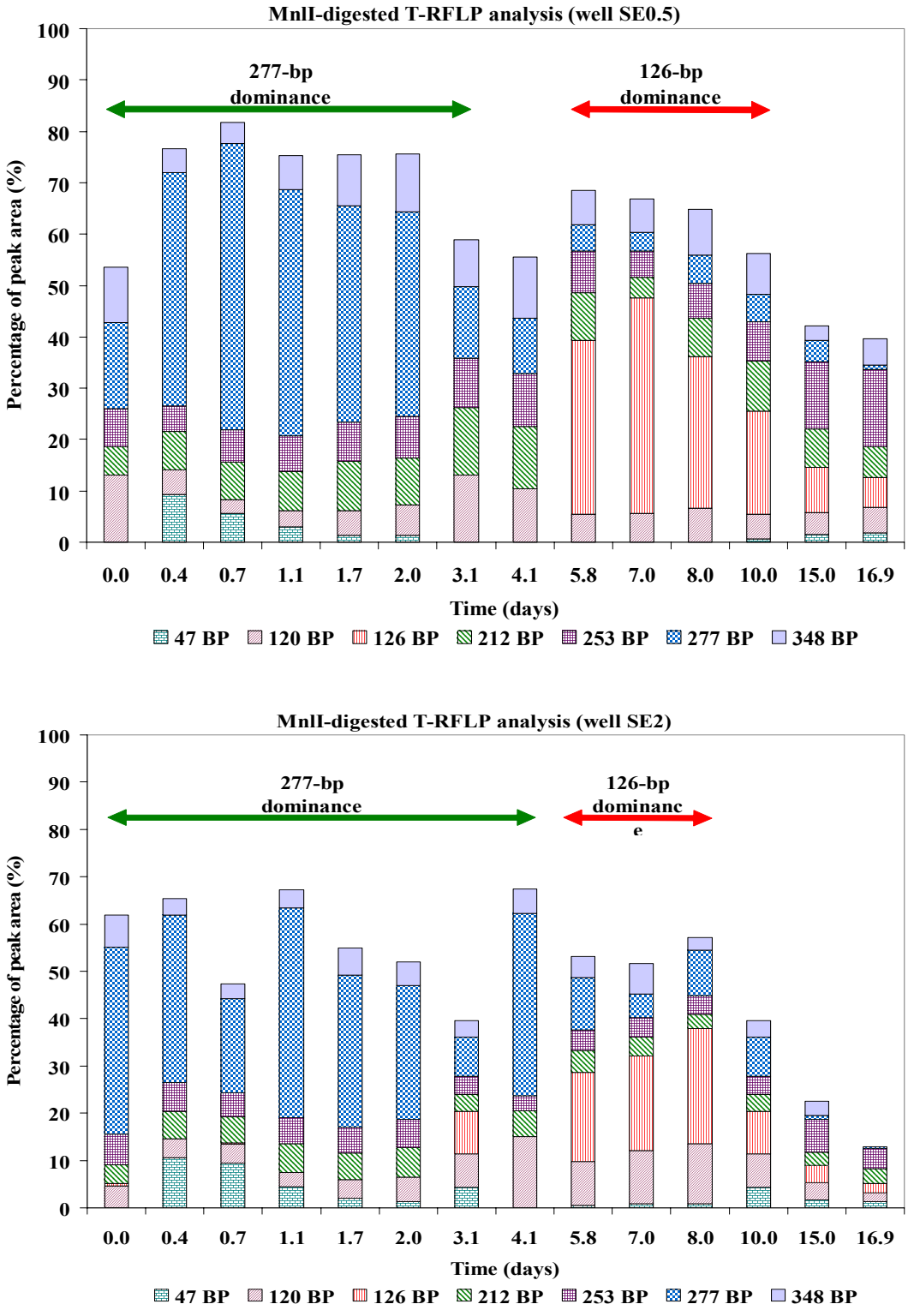


Figure C.4 Changes in the microbial community structure during the bioaugmentation test in well SE0.5 and SE2 during the bioaugmentation test conducted in December, 2003.

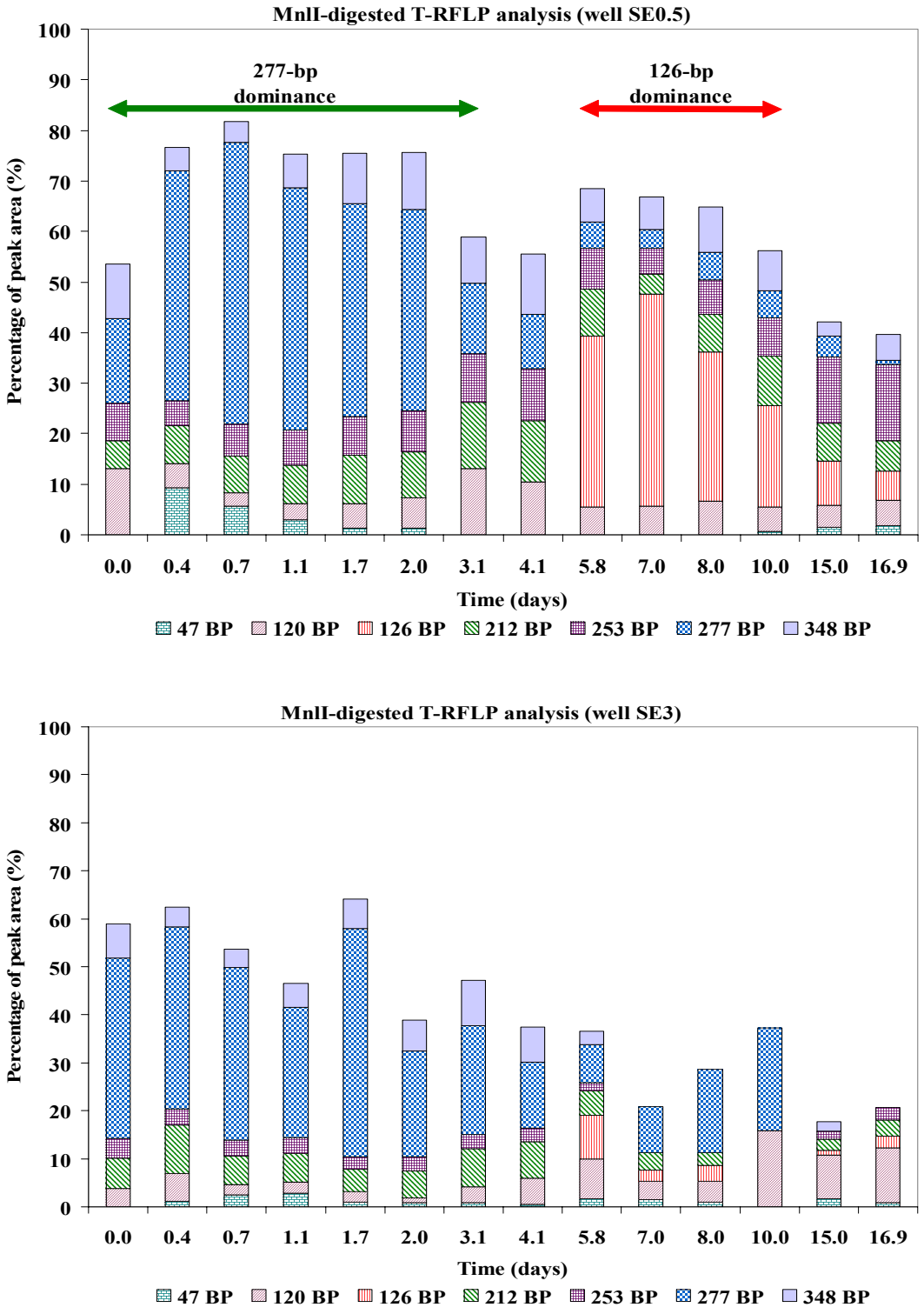
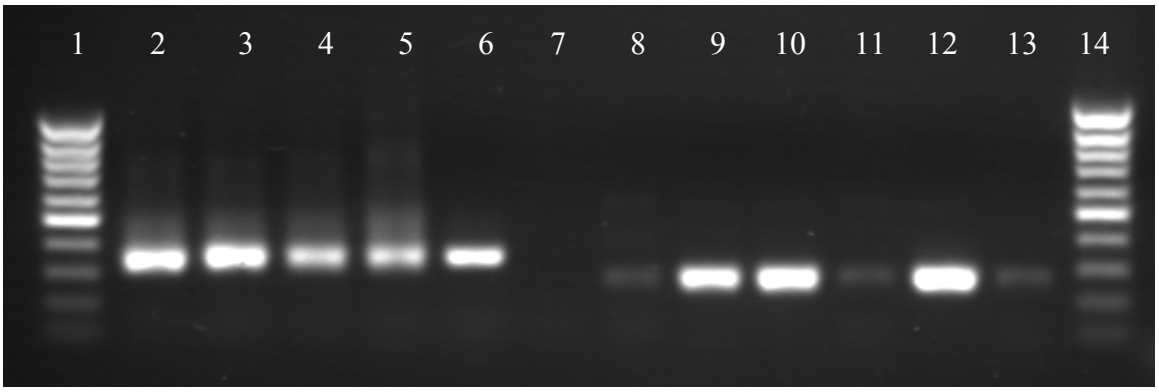


Figure C.5 Changes in the microbial community structure during the bioaugmentation test in well SE3 during the bioaugmentation test conducted in December, 2003.

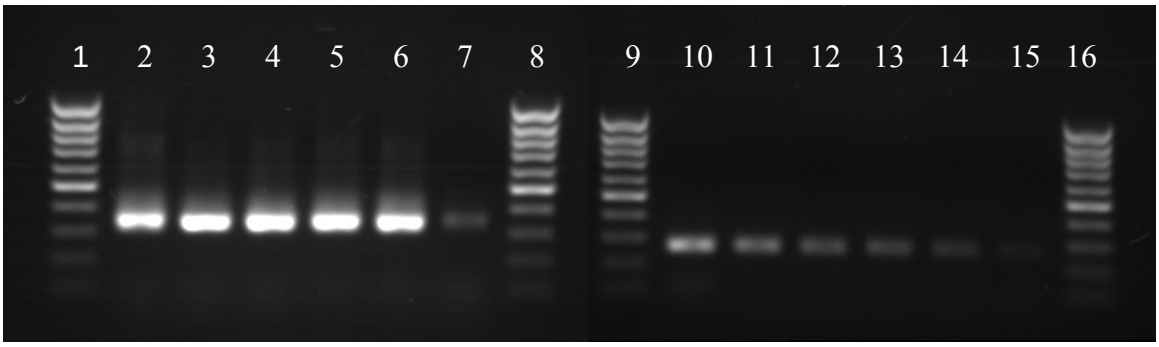
APPENDIX D

GEL-ELETROPHORESIS IMAGES



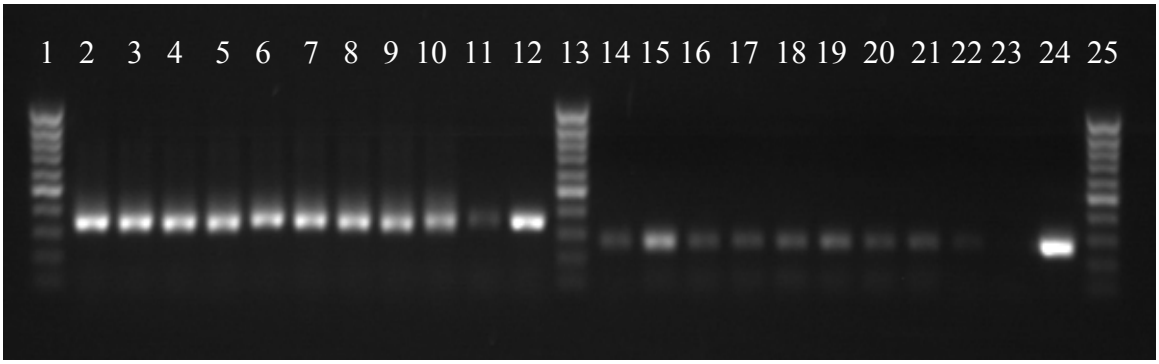
Lane	Samples / Date	Primer set	Estimated DNA concentration after amplification (ng/µl)
1	DNA mass ladder		
2	Day 0 (SE0.5)	Universal	10.1
3	Day 2 (SE0.5)	Universal	10.3
4	Day 3 (SE0.5)	Universal	7.7
5	Day 0 (SW0.5)	Universal	7.4
6	(+) control	Universal	9.5
7	(-) control	Universal	0.6
8	Day 0 (SE0.5)	183BP-specific	1.7
9	Day 2 (SE0.5)	183BP-specific	10.0
10	Day 3 (SE0.5)	183BP-specific	10.5
11	Day 0 (SW0.5)	183BP-specific	1.8
12	(+) control	183BP-specific	10.6
13	(-) control	183BP-specific	1.5
14	DNA mass ladder		

Figure D.1 Detection of strain 183BP populations in the Moffett groundwater samples taken from well SE0.5, SE1, SW0.5 and SW1 with universal bacterial primers and 183BP-specific primers during the bioaugmentation conducted in October 2003.



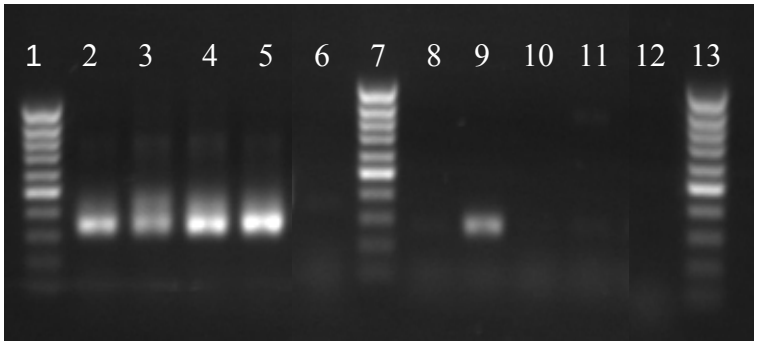
Lane	Samples / Date	Primer set	Estimated DNA concentration after amplification (ng/µl)
1	DNA mass ladder		
2	Day 5 (SE0.5)	Universal	15.1
3	Day 7 (SE0.5)	Universal	15.1
4	Day 9 (SE0.5)	Universal	15.1
5	Day 11 (SE0.5)	Universal	15.1
6	Day 11 (SE1)	Universal	15.1
7	(-) control	Universal	3.2
8	DNA mass ladder		
9	DNA mass ladder		
10	Day 5 (SE0.5)	183BP-specific	8.5
11	Day 7 (SE0.5)	183BP-specific	6.6
12	Day 9 (SE0.5)	183BP-specific	4.6
13	Day 11 (SE0.5)	183BP-specific	3.9
14	Day 11 (SE1)	183BP-specific	2.6
15	(-) control	183BP-specific	0.8
16	DNA mass ladder		

Figure D.1 (Continued) Detection of strain 183BP populations in the Moffett groundwater samples taken from well SE0.5, SE1, SW0.5 and SW1 with universal bacterial primers and 183BP-specific primers during the bioaugmentation conducted in October, 2003



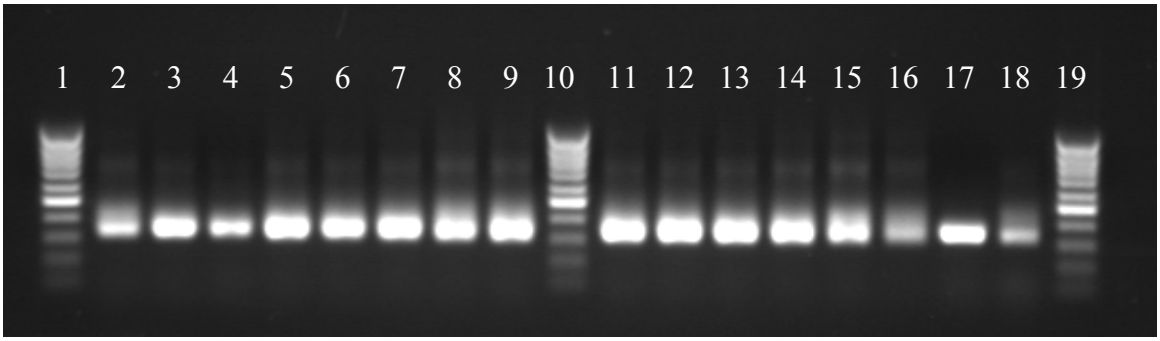
Lane	Samples / Date	Primer set	Estimated DNA concentration after amplification (ng/µl)
1	DNA mass ladder		
2	Day 13 (SE0.5)	Universal	16.4
3	Day 15 (SE0.5)	Universal	16.4
4	Day 18 (SE0.5)	Universal	16.1
5	Day 21 (SE0.5)	Universal	16.0
6	Day 13 (SE1)	Universal	15.9
7	Day 15 (SE1)	Universal	16.2
8	Day 18 (SE1)	Universal	15.9
9	Day 21 (SE1)	Universal	14.9
10	Day 21 (SW1)	Universal	12.7
11	(-) control	Universal	2.3
12	(+) control	Universal	16.4
13	DNA mass ladder		
14	Day 13 (SE0.5)	183BP-specific	3.5
15	Day 15 (SE0.5)	183BP-specific	11.7
16	Day 18 (SE0.5)	183BP-specific	3.8
17	Day 21 (SE0.5)	183BP-specific	3.8
18	Day 13 (SE1)	183BP-specific	4.5
19	Day 15 (SE1)	183BP-specific	6.4
20	Day 18 (SE1)	183BP-specific	3.5
21	Day 21 (SE1)	183BP-specific	3.8
22	Day 21 (SW1)	183BP-specific	1.4
23	(-) control	183BP-specific	0.2
24	(+) control	183BP-specific	18.3
25	DNA mass ladder		

Figure D.1 (Continued) Detection of strain 183BP populations in the Moffett groundwater samples taken from well SE0.5, SE1, SW0.5 and SW1 with universal bacterial primers and 183BP-specific primers during the bioaugmentation conducted in October, 2003



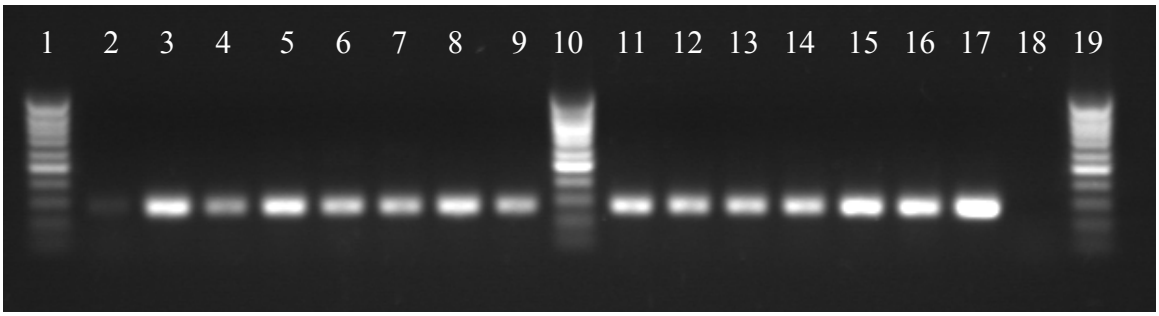
Lane	Samples / Date	Primer set	Estimated DNA concentration after amplification (ng/µl)
1	DNA mass ladder		
2	Day 27 (SE0.5)	Universal	10.6
3	Day 30 (SE0.5)	Universal	8.5
4	Day 27 (SE1)	Universal	13.4
5	Day 30 (SW1)	Universal	13.5
6	(-) control	Universal	0.8
7	DNA mass ladder		
8	Day 27 (SE0.5)	183BP-specific	0.8
9	Day 30 (SE0.5)	183BP-specific	6.2
10	Day 27 (SE1)	183BP-specific	0.7
11	Day 30 (SW1)	183BP-specific	0.9
12	(-) control	183BP-specific	0.4
13	DNA mass ladder		

Figure D.1 (Continued) Detection of strain 183BP populations in the Moffett groundwater samples taken from well SE0.5, SE1, SW0.5 and SW1 with universal bacterial primers and 183BP-specific primers during the bioaugmentation conducted in October, 2003



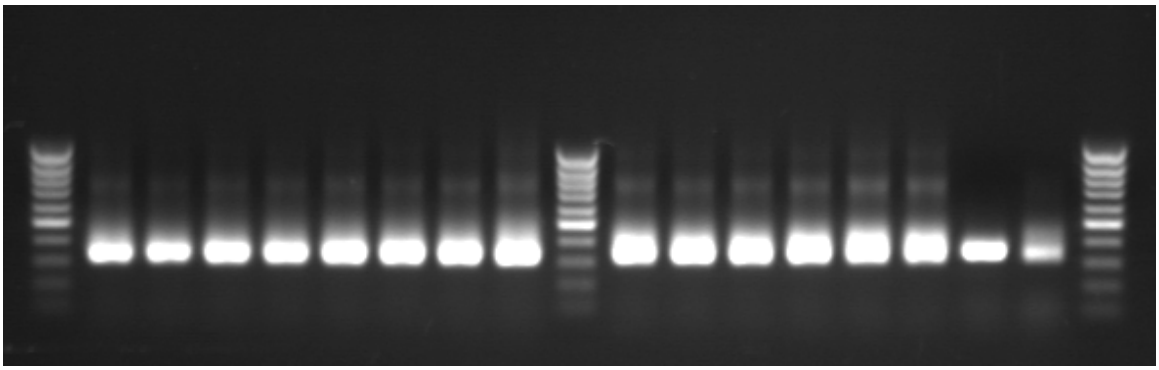
Lane	Samples / Date	Primer set	Estimated DNA concentration after amplification (ng/µl)
1	DNA mass ladder		
2	Day 0	Universal	10.7
3	Day 0.4	Universal	11.2
4	Day 0.7	Universal	10.9
5	Day 1.1	Universal	11.2
6	Day 1.7	Universal	11.1
7	Day 2	Universal	11.2
8	Day 3.1	Universal	12.0
9	Day 4.1	Universal	15.6
10	DNA mass ladder		
11	Day 5.8	Universal	11.1
12	Day 7.0	Universal	11.3
13	Day 8.0	Universal	11.3
14	Day 10	Universal	11.2
15	Day 15.0	Universal	11.1
16	Day 16.9	Universal	8.7
17	(+) control	Universal	10.9
18	(-) control	Universal	5.3
19	DNA mass ladder		

Figure D.2 Detection of strain 183BP populations in the Moffett groundwater samples taken from well SE0.5 with universal bacterial primers and 183BP-specific primers during the bioaugmentation conducted in December, 2003



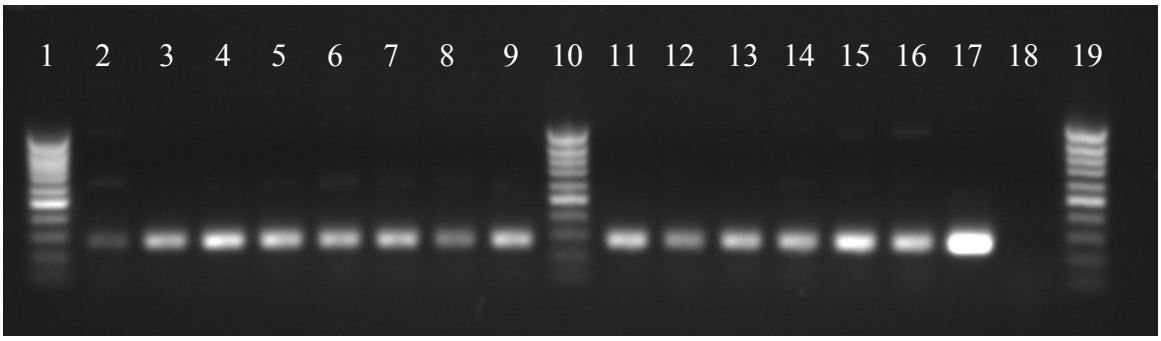
Lane	Samples / Date	Primer set	Estimated DNA concentration after amplification (ng/µl)
1	DNA mass ladder		
2	Day 0	183BP-specific	2.3
3	Day 0.4	183BP-specific	10.3
4	Day 0.7	183BP-specific	6.3
5	Day 1.1	183BP-specific	10.4
6	Day 1.7	183BP-specific	8.5
7	Day 2	183BP-specific	8.2
8	Day 3.1	183BP-specific	12.0
9	Day 4.1	183BP-specific	15.6
10	DNA mass ladder		
11	Day 5.8	183BP-specific	7.7
12	Day 7.0	183BP-specific	7.3
13	Day 8.0	183BP-specific	7.7
14	Day 10	183BP-specific	8.3
15	Day 15.0	183BP-specific	9.7
16	Day 16.9	183BP-specific	9.9
17	(+) control	183BP-specific	10.6
18	(-) control	183BP-specific	0.4
19	DNA mass ladder		

Figure D.2 (Continued) Detection of strain 183BP populations in the Moffett groundwater samples taken from well SE0.5 with universal bacterial primers and 183BP-specific primers during the bioaugmentation conducted in December, 2003



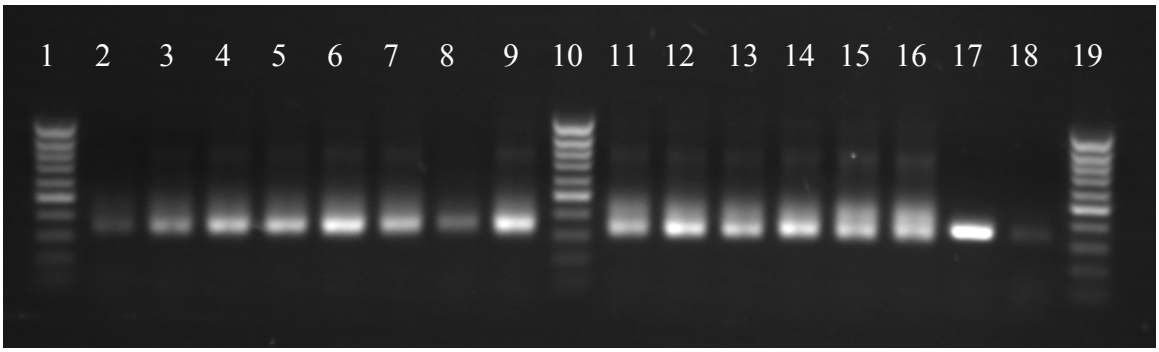
Lane	Samples / Date	Primer set	Estimated DNA concentration after amplification (ng/µl)
1	DNA mass ladder		
2	Day 0	Universal	10.3
3	Day 0.4	Universal	10.3
4	Day 0.7	Universal	10.3
5	Day 1.1	Universal	10.3
6	Day 1.7	Universal	10.3
7	Day 2	Universal	10.3
8	Day 3.1	Universal	10.3
9	Day 4.1	Universal	10.3
10	DNA mass ladder		
11	Day 5.8	Universal	13.1
12	Day 7.0	Universal	13.0
13	Day 8.0	Universal	13.1
14	Day 10	Universal	13.1
15	Day 15.0	Universal	13.1
16	Day 16.9	Universal	13.1
17	(+) control	Universal	13.0
18	(-) control	Universal	6.2
19	DNA mass ladder		

Figure D.3 Detection of strain 183BP populations in the Moffett groundwater samples taken from well SE1 with universal bacterial primers and 183BP-specific primers during the bioaugmentation conducted in December, 2003



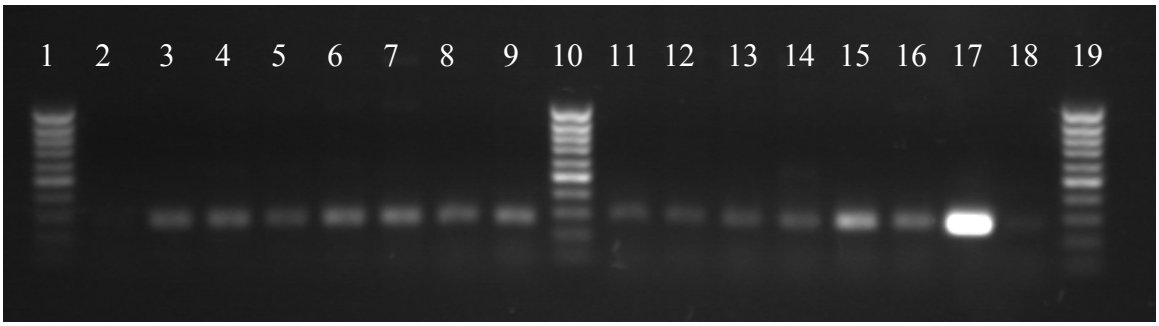
Lane	Samples / Date	Primer set	Estimated DNA concentration after amplification (ng/µl)
1	DNA mass ladder		
2	Day 0	183BP-specific	2.7
3	Day 0.4	183BP-specific	7.8
4	Day 0.7	183BP-specific	10.7
5	Day 1.1	183BP-specific	9.2
6	Day 1.7	183BP-specific	8.1
7	Day 2	183BP-specific	8.1
8	Day 3.1	183BP-specific	12.0
9	Day 4.1	183BP-specific	15.6
10	DNA mass ladder		
11	Day 5.8	183BP-specific	10.1
12	Day 7.0	183BP-specific	6.8
13	Day 8.0	183BP-specific	9.9
14	Day 10	183BP-specific	10.0
15	Day 15.0	183BP-specific	13.8
16	Day 16.9	183BP-specific	11.8
17	(+) control	183BP-specific	16.7
18	(-) control	183BP-specific	0.2
19	DNA mass ladder		

Figure D.3 (Continued) Detection of strain 183BP populations in the Moffett groundwater samples taken from well SE1 with universal bacterial primers and 183BP-specific primers during the bioaugmentation conducted in December, 2003



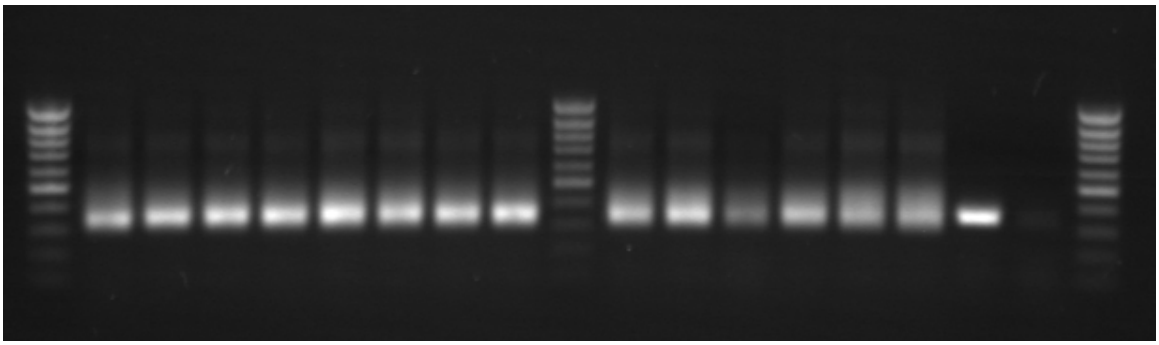
Lane	Samples / Date	Primer set	Estimated DNA concentration after amplification (ng/µl)
1	DNA mass ladder		
2	Day 0	Universal	3.8
3	Day 0.4	Universal	9.4
4	Day 0.7	Universal	14.1
5	Day 1.1	Universal	14.5
6	Day 1.7	Universal	17.1
7	Day 2	Universal	13.2
8	Day 3.1	Universal	12.0
9	Day 4.1	Universal	15.6
10	DNA mass ladder		
11	Day 5.8	Universal	10.4
12	Day 7.0	Universal	14.8
13	Day 8.0	Universal	12.6
14	Day 10	Universal	15.1
15	Day 15.0	Universal	13.3
16	Day 16.9	Universal	13.3
17	(+) control	Universal	15.2
18	(-) control	Universal	2.1
19	DNA mass ladder		

Figure D.4 Detection of strain 183BP populations in the Moffett groundwater samples taken from well SE1.5 with universal bacterial primers and 183BP-specific primers during the bioaugmentation conducted in December, 2003



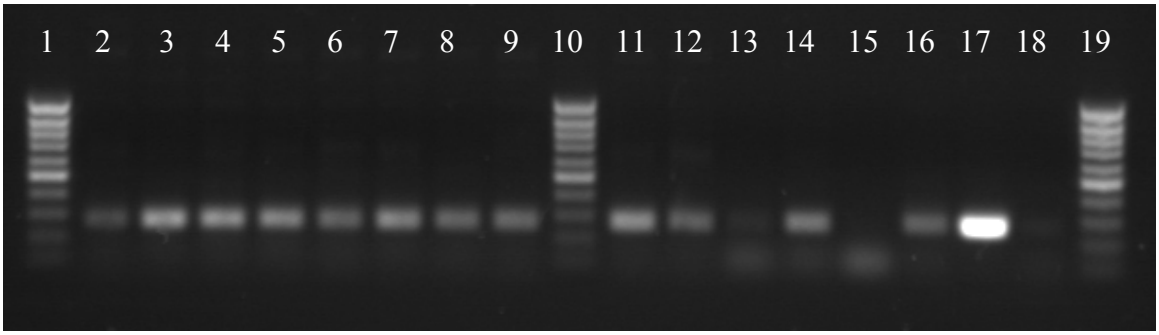
Lane	Samples / Date	Primer set	Estimated DNA concentration after amplification (ng/µl)
1	DNA mass ladder		
2	Day 0	183BP-specific	2.4
3	Day 0.4	183BP-specific	4.6
4	Day 0.7	183BP-specific	5.0
5	Day 1.1	183BP-specific	4.2
6	Day 1.7	183BP-specific	5.8
7	Day 2	183BP-specific	6.0
8	Day 3.1	183BP-specific	12.0
9	Day 4.1	183BP-specific	15.6
10	DNA mass ladder		
11	Day 5.8	183BP-specific	2.3
12	Day 7.0	183BP-specific	2.0
13	Day 8.0	183BP-specific	2.7
14	Day 10	183BP-specific	3.3
15	Day 15.0	183BP-specific	8.3
16	Day 16.9	183BP-specific	4.8
17	(+) control	183BP-specific	17.3
18	(-) control	183BP-specific	1.2
19	DNA mass ladder		

Figure D.4 (Continued) Detection of strain 183BP populations in the Moffett groundwater samples taken from well SE1.5 with universal bacterial primers and 183BP-specific primers during the bioaugmentation conducted in December, 2003



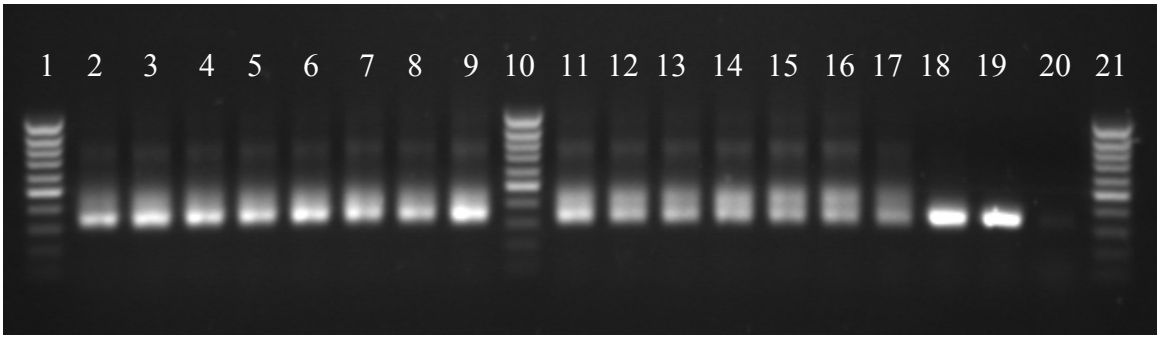
Lane	Samples / Date	Primer set	Estimated DNA concentration after amplification (ng/µl)
1	DNA mass ladder		
2	Day 0	Universal	14.4
3	Day 0.4	Universal	17.4
4	Day 0.7	Universal	18.4
5	Day 1.1	Universal	18.6
6	Day 1.7	Universal	19.4
7	Day 2	Universal	17.8
8	Day 3.1	Universal	12.0
9	Day 4.1	Universal	15.6
10	DNA mass ladder		
11	Day 5.8	Universal	14.0
12	Day 7.0	Universal	17.0
13	Day 8.0	Universal	9.3
14	Day 10	Universal	15.5
15	Day 15.0	Universal	14.1
16	Day 16.9	Universal	14.6
17	(+) control	Universal	17.1
18	(-) control	Universal	2.3
19	DNA mass ladder		

Figure D.5 Detection of strain 183BP populations in the Moffett groundwater samples taken from well SE2 with universal bacterial primers and 183BP-specific primers during the bioaugmentation conducted in December, 2003



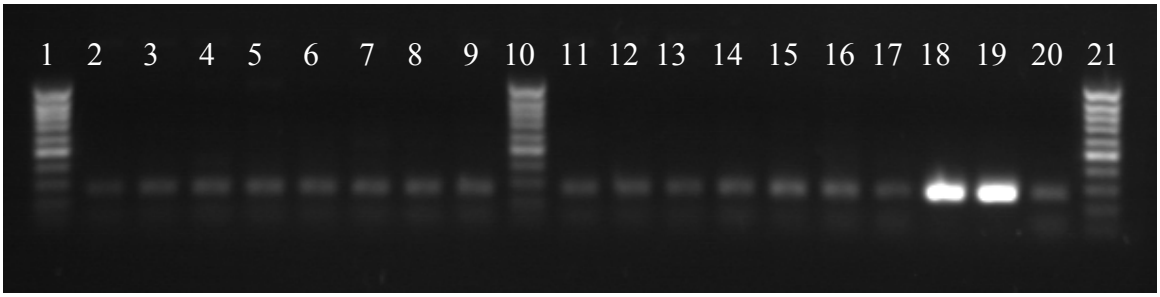
Lane	Samples / Date	Primer set	Estimated DNA concentration after amplification (ng/µl)
1	DNA mass ladder		
2	Day 0	183BP-specific	3.9
3	Day 0.4	183BP-specific	9.2
4	Day 0.7	183BP-specific	8.7
5	Day 1.1	183BP-specific	7.7
6	Day 1.7	183BP-specific	5.7
7	Day 2	183BP-specific	7.5
8	Day 3.1	183BP-specific	12.0
9	Day 4.1	183BP-specific	15.6
10	DNA mass ladder		
11	Day 5.8	183BP-specific	7.4
12	Day 7.0	183BP-specific	4.8
13	Day 8.0	183BP-specific	1.9
14	Day 10	183BP-specific	6.4
15	Day 15.0	183BP-specific	1.3
16	Day 16.9	183BP-specific	5.3
17	(+) control	183BP-specific	17.9
18	(-) control	183BP-specific	1.6
19	DNA mass ladder		

Figure D.5 (Continued) Detection of strain 183BP populations in the Moffett groundwater samples taken from well SE2 with universal bacterial primers and 183BP-specific primers during the bioaugmentation conducted in December, 2003



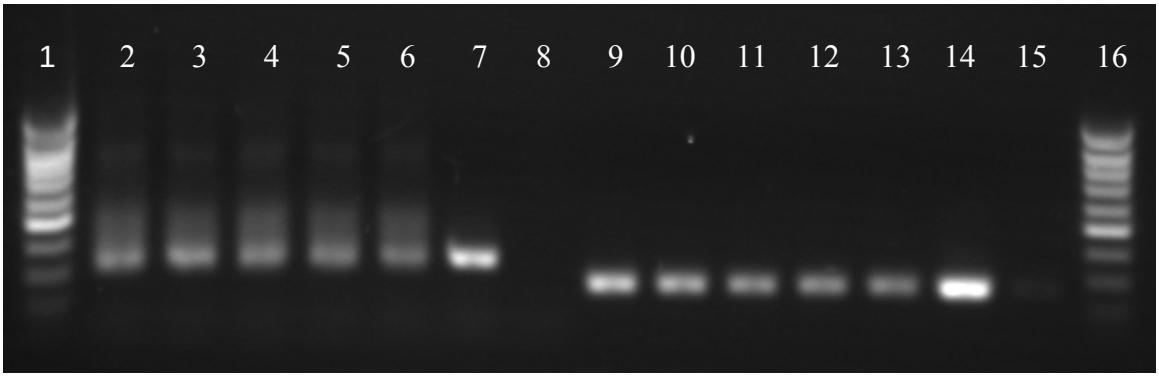
Lane	Samples / Date	Primer set	Estimated DNA concentration after amplification (ng/µl)
1	DNA mass ladder		
2	Day 0 (SE3)	Universal	11.7
3	Day 0.4 (SE3)	Universal	13.1
4	Day 0.7 (SE3)	Universal	13.2
5	Day 1.1 (SE3)	Universal	13.0
6	Day 1.7 (SE3)	Universal	13.2
7	Day 2 (SE3)	Universal	12.6
8	Day 3.1 (SE3)	Universal	11.6
9	Day 4.1 (SE3)	Universal	13.1
10	DNA mass ladder		
11	Day 5.8 (SE3)	Universal	11.8
12	Day 7.0 (SE3)	Universal	10.3
13	Day 8.0 (SE3)	Universal	10.2
14	Day 10 (SE3)	Universal	10.8
15	Day 15.0 (SE3)	Universal	10.2
16	Day 16.9 (SE3)	Universal	10.0
17	Day 16.9 (SW0.5)	Universal	8.3
18	Bioaugmented culture	Universal	13.4
19	(+) control	Universal	13.3
20	(-) control	Universal	1.2
21	DNA mass ladder		

Figure D.6 Detection of strain 183BP populations in the Moffett groundwater samples taken from well SE3 with universal bacterial primers and 183BP-specific primers during the bioaugmentation conducted in December, 2003



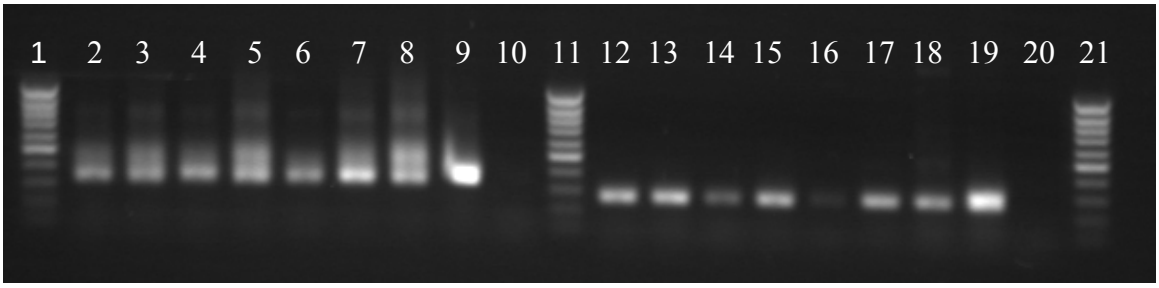
Lane	Samples / Date	Primer set	Estimated DNA concentration after amplification (ng/µl)
1	DNA mass ladder		
2	Day 0 (SE3)	183BP-specific	2.4
3	Day 0.4 (SE3)	183BP-specific	3.2
4	Day 0.7 (SE3)	183BP-specific	3.8
5	Day 1.1 (SE3)	183BP-specific	4.1
6	Day 1.7 (SE3)	183BP-specific	3.9
7	Day 2 (SE3)	183BP-specific	4.0
8	Day 3.1 (SE3)	183BP-specific	5.2
9	Day 4.1 (SE3)	183BP-specific	5.6
10	DNA mass ladder		
11	Day 5.8 (SE3)	183BP-specific	3.0
12	Day 7.0 (SE3)	183BP-specific	3.3
13	Day 8.0 (SE3)	183BP-specific	3.1
14	Day 10 (SE3)	183BP-specific	3.7
15	Day 15.0 (SE3)	183BP-specific	4.6
16	Day 16.9 (SE3)	183BP-specific	4.3
17	Day 16.9 (SW0.5)	183BP-specific	3.4
18	Bioaugmented culture	183BP-specific	14.5
19	(+) control	183BP-specific	14.6
20	(-) control	183BP-specific	3.0
21	DNA mass ladder		

Figure D.6 (Continued) Detection of strain 183BP populations in the Moffett groundwater samples taken from well SE3 with universal bacterial primers and 183BP-specific primers during the bioaugmentation conducted in December, 2003



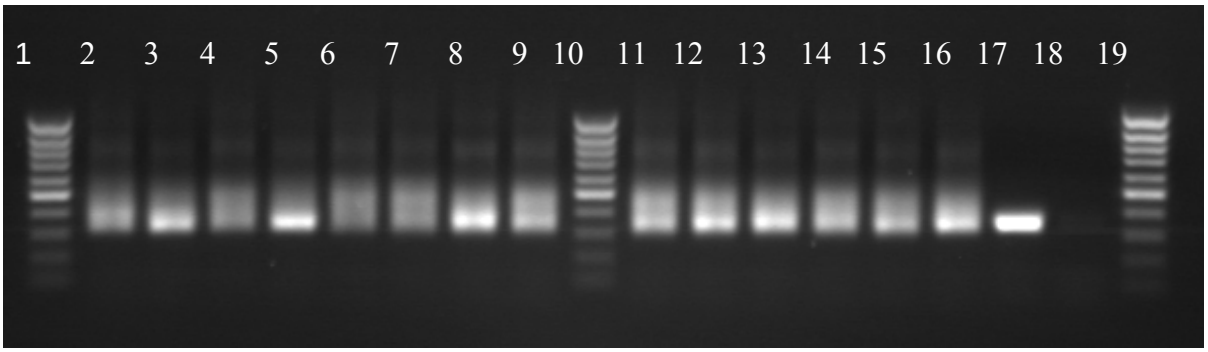
Lane	Samples	Date	Primer set	Estimated DNA concentration after amplification (ng/µl)
1	DNA mass ladder			
2	B1	Day 37	Universal	6.7
3	B2	Day 44	Universal	7.4
4	B3	Day 48	Universal	6.6
5	B4	Day 51	Universal	6.4
6	B5	Day 55	Universal	5.9
7	(+) control		Universal	11.3
8	(-) control		Universal	1.5
9	DNA mass ladder			
10	B1	Day 37	183BP-specific	10.5
11	B2	Day 44	183BP-specific	8.5
12	B3	Day 48	183BP-specific	6.8
13	B4	Day 51	183BP-specific	6.8
14	B5	Day 55	183BP-specific	6.2
15	(+) control		183BP-specific	14.1
16	(-) control		183BP-specific	1.8

Figure D.7 Detection of strain 183BP populations in the soil column effluent samples with universal bacterial primers and 183BP-specific primers



Lane	Samples	Date	Primer set	Estimated DNA concentration after amplification (ng/µl)
1	DNA mass ladder			
2	B6	Day 58	Universal	9.2
3	B7	Day 62	Universal	10.1
4	B8	Day 65	Universal	11.3
5	B9	Day 69	Universal	12.9
6	B10	Day 72	Universal	10.4
7	B11	Day 76	Universal	17.3
8	B12	Day 83	Universal	14.0
9	B13	Day 86	Universal	19.8
10	(-) control		Universal	0.9
11	DNA mass ladder			
12	B6	Day 58	183BP-specific	10.8
13	B7	Day 62	183BP-specific	12.5
14	B8	Day 65	183BP-specific	5.4
15	B9	Day 69	183BP-specific	11.6
16	B10	Day 72	183BP-specific	2.3
17	B11	Day 76	183BP-specific	11.1
18	B12	Day 83	183BP-specific	10.1
19	B13	Day 86	183BP-specific	18.4
20	(-) control		183BP-specific	0.5
21	DNA mass ladder			

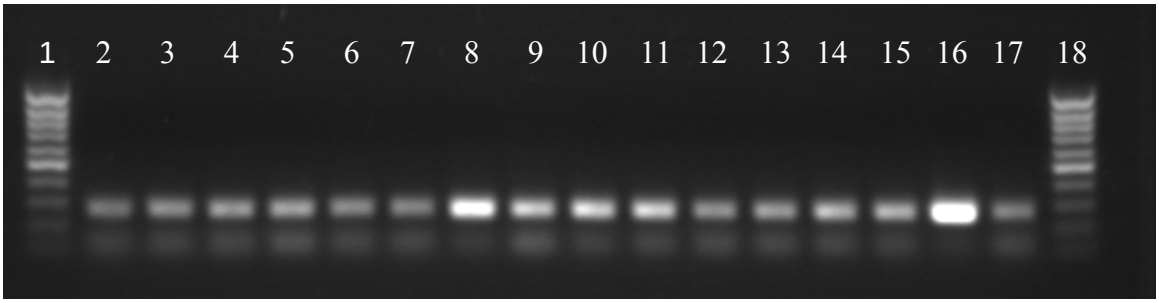
Figure D.7 (Continued) Detection of strain 183BP populations in the soil column effluent samples with universal bacterial primers and 183BP-specific primers



Lane	Samples	Date	Primer set	Estimated DNA concentration after amplification (ng/µl)
1	DNA mass ladder			
2	B14	Day 90	Universal	9.0
3	B15	Day 93	Universal	14.0
4	B16	Day 97	Universal	8.8
5	B17	Day 100	Universal	15.7
6	B18	Day 104	Universal	7.4
7	B19	Day 107	Universal	8.6
8	B20	Day 111	Universal	12.0
9	B21	Day 118	Universal	15.6
10	DNA mass ladder			
11	B22	Day 119	Universal	9.4
12	B23	Day 121	Universal	11.9
13	B24	Day 125	Universal	12.7
14	B25	Day 128	Universal	10.3
15	B26	Day 132	Universal	11.1
16	B27	Day 134	Universal	13.1
17	(+) control		Universal	14.3
18	(-) control		Universal	1.6

19 DNA mass ladder

Figure D.7 (Continued) Detection of strain 183BP populations in the soil column effluent samples with universal bacterial primers and 183BP-specific primers



Lane	Samples	Date	Primer set	Estimated DNA concentration after amplification (ng/µl)
1	DNA mass ladder			
2	B14	Day 90	183BP-specific	6.2
3	B15	Day 93	183BP-specific	7.9
4	B16	Day 97	183BP-specific	9.1
5	B17	Day 100	183BP-specific	9.5
6	B18	Day 104	183BP-specific	7.6
7	B19	Day 107	183BP-specific	7.0
8	B20	Day 111	183BP-specific	12.0
9	B21	Day 118	183BP-specific	15.6
10	B22	Day 119	183BP-specific	10.9
11	B23	Day 121	183BP-specific	11.5
12	B24	Day 125	183BP-specific	8.1
13	B25	Day 128	183BP-specific	8.6
14	B26	Day 132	183BP-specific	10.9
15	B27	Day 134	183BP-specific	10.7
16	(+) control		183BP-specific	20.5
17	(-) control		183BP-specific	4.4
18	DNA mass ladder			

Figure D.7 (Continued) Detection of strain 183BP populations in the soil column effluent samples with universal bacterial primers and 183BP-specific primers